

## More talk to help Europe's money

**Last week's contest for the leadership of Britain's Conservative party was a missed chance by both sides to say what is right and wrong about plans for a common currency**

BRITAIN'S domestic contest for leadership of the Conservative party begun, last week, by an election by Conservative MPs in which the candidates were the prime minister, Mr John Major, and a previous member of his cabinet, Mr John Redwood, was driven by the strong opinion of many of the same MPs that Britain's integration into Europe has gone too far. It may therefore have been thought that the several days of debate preceding last Tuesday's vote would have at least clarified the issues from which discontent seems perennially to spring. But the opposite is the case. Redwood, the challenger in the election, dutifully said that a government of which he was the head would publicly set its face against membership of the proposed European currency system. The prime minister and his friends insist, as they have done since the negotiation of the Maastricht Treaty, that the time to decide is not now, but when the details of the common currency have been worked out. Neither side has said much to enlighten those who will vote in the general election there must be in less than two years.

That is a shabby and unconstructive way to carry on. The truth is that the question of the common currency is still undecided. Whatever the Maastricht Treaty says, there is no way in which other major members of the European Union (EU) than Britain will accept the proposals as they have been outlined. The central issue is not, of course, whether the local coinage should continue to carry representations of local dignitaries' heads, but whether Europe should now move towards a common monetary policy, and whether national fiscal policies should be subordinate to that larger framework. Germany has long been anxious that the plan will simply undermine its prudent management of its own currency in the past three decades. France also has second thoughts, as President Jacques Chirac told Major only the other day. And there is widespread disbelief that Italy will be able to reduce its annual budget deficit to less than 3 per cent of gross domestic product by the time the scheme is due to operate, perhaps in 1999.

The outline of the Maastricht scheme is simple. There is to be a European Monetary Institute that will function as a central bank independent of political control. Its function will be to manage monetary policy, in which the chief instrument is the determination of interest rates, for Europe as a whole. It would also become the lender of last resort to national governments. One danger in the system is that members of the EU choosing to operate with, say, grossly unbalanced budgets will be able to export the inflation such

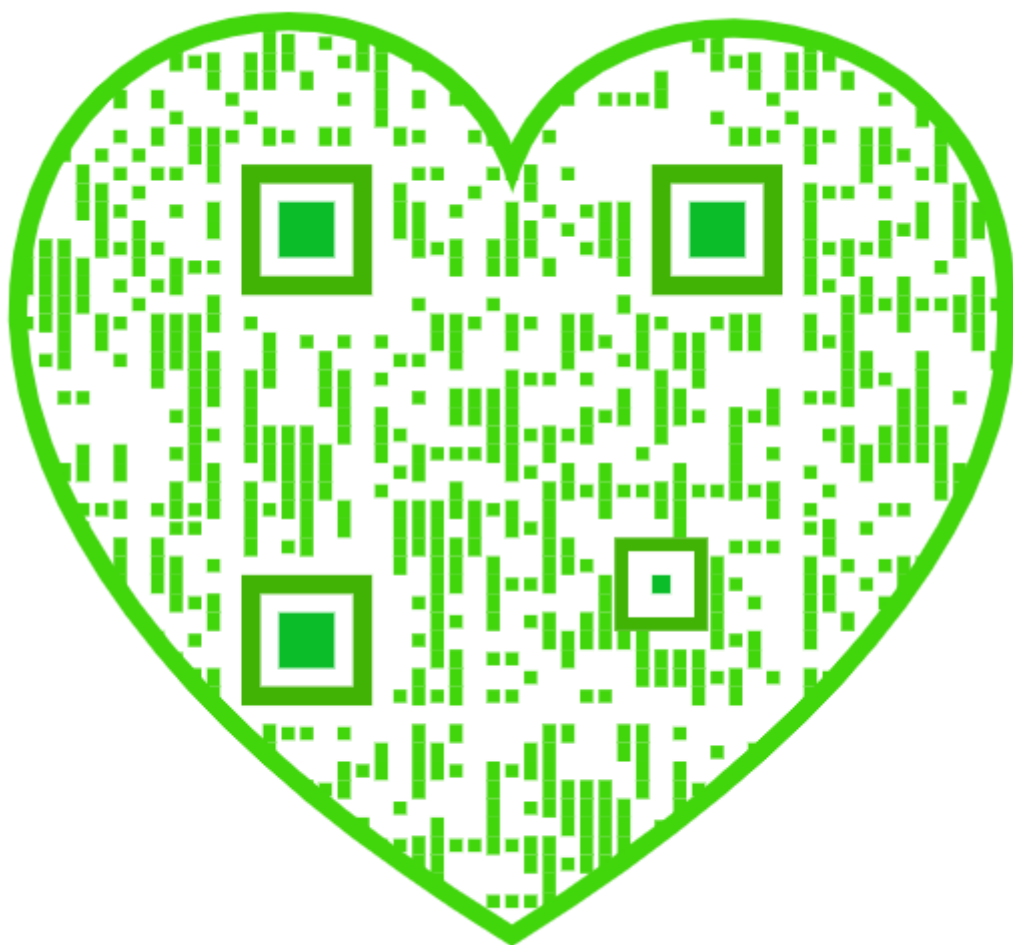
a practice would normally engender to the rest of Europe. To guard against that, Maastricht proposes a system by which countries would be fined by the central bank in a draconian way, which is politically unworkable. Another danger is that the system will be costly; relatively impoverished parts of Europe will qualify for regional aid, whereas, in a fully integrated economy, people would follow prosperity to other regions. A third is that national governments would be inhibited from using fiscal policy to cure domestic ills, unemployment for example.

Given these imponderables, it is natural that there should be general anxiety about the proposed quick march to a common currency. In many ways, it is foolhardy to pursue the goal without a better and wider understanding of how the difficulties would be overcome. That, in essence, is Chirac's point the other day. At least two courses might be followed. The Benelux members of the European Union appear to be anxious to get on with the project; why not encourage them to do so? That way, there would be some practical experience that might even encourage the others. But there is a more urgent need for more public talk about the details of a common currency. By sticking to broad generalities, the participants in last week's leadership election missed an opportunity that could only have strengthened their respective cases. □

## More trade bilateralism

**On the heels of an aborted trade war with Japan, the United States is still hankering after bilateralism.**

MORE by good luck than good management, the advertised trade dispute between the United States and Japan over their mutual trade in motor-cars and parts thereof has been averted. For practical purposes, the United States has accepted that it cannot ask the government of Japan to guarantee a predetermined volume of US exports every year, and has instead accepted the Japanese government's offer to remind its own traders of their freedom to sell US cars and components if they so wish. Plainly the outcome is neither that which the Clinton administration set out to secure, nor that which President Bill Clinton claimed in the aftermath of the negotiations. Will he and his advisers now appreciate that it might have been more effective to have followed the rules of the international trading system, and to have taken



the complaints of US car manufacturers to the World Trade Organization (WTO)?

The question must be asked because it has already arisen, if in a different context. The WTO is the inheritor of the General Agreement on Tariffs and Trade (GATT), within which framework the principal trading nation had been negotiating a complicated package of measures aimed at further liberalization of the trading regime. One component of that negotiation was the notion that international trade in services should be liberalized, in the sense that companies offering potential customers services as different as computer management and life insurance should be free to offer them internationally, within the company of the countries signing on to a negotiated arrangement. The completion of that arrangement was delegated to WTO.

Superficially, at least, the United States is again the chief source of trouble. From services in general, the scope of the present negotiations has been narrowed to include only financial services — banking, insurance and the marketing of financial securities such as stocks and bonds. But the precedents set in these negotiations will no doubt apply to the more technical issues that come later on the agenda, telecommunications in particular. The difficulty is that the spirit of the GATT (and WTO) rules does not prevent countries from keeping overseas companies out of their domestic markets, which they may do by means of tariffs, but do require that they do not discriminate between overseas suppliers on the grounds of nationality.

That implies that country A may decide to open its domestic market for some commodity or service to all comers, and then find that country B is less liberal by imposing a (non-discriminatory) tariff on imported goods or by imposing impossibly tough conditions on those who would sell services. The underlying principle is that these asymmetries will not matter in the long run. Consumers in country A will benefit immediately from competition between a diversity of suppliers, domestic as well as foreign, while consumers in country B will eventually come to envy the benefits people elsewhere enjoy, and will make their government change its policy. The benefit accruing to those who live in country A is not, of course, to a first approximation, diminished by the policy of country B, however illiberal it may be.

What has now happened at the WTO is that the United States is insisting that it will discriminate against others wishing to sell it financial services who fail to offer the same liberality as it offers to others. Japanese banks will be free to operate in the United States only if US banks can set up in Tokyo, for example. On the face of things, this looks fair. And it is inevitable that the WTO pact on financial services will not be signed unless the 30 negotiating countries are satisfied with what their potential partners offer. But to go so far as to insist that all trading partners should offer exactly the same liberal package is to ask more than is necessary. It is also an awkward echo of the automobile dispute. For the past quarter of a century, the United States has been the chief force impelling the world towards free trade, mostly by its support for international institutions. It is not easy to understand why its enthusiasm seems to have evaporated □

## A broken embargo

**Journalists who break agreed embargoes damage not only themselves but also their profession.**

THIS journal follows many (but not all) others in operating a strict policy on the release of information about to be published. As authors know well enough, they are told when a manuscript is accepted for publication that they should not make the information public until the day and even the hour of publication. Part of the explanation of that policy is that *Nature* does not wish its own readers to be scooped by advance publicity for an important development. And much of what passes for important news is not readily digested by busy journalists, for which reason *Nature* distributes each week a readable press digest, again with a strict embargo linked with the date of publication. If they wish, journalists can be sent an advance copy of an article in which they are particularly interested. The service appears to be welcomed by journalists, who seem to find it helpful.

It goes without saying that this practice is not meant to prevent researchers saying what they wish at scientific meetings. The rule is that if they give an account of their most recent work and happen to be heard by a journalist, they have a duty of courtesy to him or her to answer intelligent questions after their presentation, whether or not the person concerned is planning to write something about the new development. But researchers with an article in the press should stop short of handing over a copy of the proofs or of being filmed for a television programme without a firm assurance that the embargo will be respected. That, too, seems to be accepted by journalists and researchers alike.

Except, it appears, for Mr Tim Friend, a journalist with the daily *USA Today*. One of highlights of last week's issue of *Nature* was the article by R. Sherrington *et al.* describing mutations in a gene apparently responsible for some early-onset Alzheimer's disease (*Nature* **375**, 754–760; 1995). On Tuesday last week, one of the authors was giving evidence to a US congressional committee seeking opinions on the importance of basic research, and offered the characterization of this gene as one of the benefits. The reference was picked up by ABC News and eventually mentioned in the television network's 7 p.m. news bulletin. No less a person than Mr Peter Jennings referred to the discovery and its impending publication, adding that the authors had nothing more to say at that stage.

Unconstrained, *USA Today* published a full account of the research the following day. Friend quoted Jennings as the excuse for this deliberate breaking of an agreed embargo, saying that the fault lay with Jennings and not with him. In this journal's view, that is disingenuous. Indeed, Jennings as the author had said no more than had the panel on *Nature*'s contents page the previous week. *Nature* therefore has no choice but to remove Friend and *USA Today* from its press distribution list. While it may seem to them that they are being penalized unfairly for breaking bureaucratic rules, their real disservice is to their fellow-journalists. □

# Environmental groups get Supreme Court boost on endangered species

**Washington.** In an increasingly rare victory for environmental advocates, the US Supreme Court last week ruled that destroying wildlife habitat constitutes a type of harm to protected species, and can therefore be prohibited by law.

The 6:3 decision overturns a lower court ruling that the Endangered Species Act (ESA) forbids only direct, intentional harm to animals and plants, a ruling that would have negated federal regulations that are intended to prevent private property owners from destroying habitat.

The justices looked to Webster's *Third New International Dictionary*, among other sources, to help make their decision, which focused on the fate of the northern spotted owl in Oregon. Writing for the majority, Justice John Paul Stevens held that the dictionary definition of the verb form of 'harm' is "to cause hurt or damage to; injure". In the context of the act, he said, that definition "naturally encompasses habitat modification that results in actual injury or death to members of an endangered or threatened species".

The decision, known as *Babbitt vs Sweet Home Chapter of Communities for a Great Oregon*, confirms — perhaps for good — the legal basis of the ESA, which comes before Congress for reauthorization this year. It follows on the heels of a strong

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASON

**Sitting comfortably: habitat of the northern spotted owl now has judicial protection.**

scientific endorsement of the act by the National Academy of Sciences in May (see *Nature*, 375, 349; 1995).

Yet environmentalists were hardly in a mood to celebrate. Two days earlier, the House of Representatives Appropriations Committee had voted to slash the budgets of

the two federal agencies responsible for biological research within the Department of Interior, which administers the ESA.

The committee deleted all money (about \$12.5 million) in the Fish and Wildlife Service's 1996 budget for listing or 'pre-listing' new threatened or endangered species. Such listings are already under a moratorium imposed by Congress — deleting next year's funding would simply extend the moratorium and stop the ESA in its tracks.

The committee also effectively abolished the new National Biological Service (NBS) by transferring its functions to the US Geological Survey (USGS) and cutting its budget by one third, to \$113 million. The beleaguered director of NBS, Ron Pulliam, released a statement calling the vote a "decision to throw the baby out with the bath water", and saying that the merger of NBS with the USGS "reflects a misunderstanding of the basic responsibilities of the two agencies".

In a more-than-usually pointed message, Pulliam repeated an argument he has made unsuccessfully in the Congress, namely that research by the biological survey serves not only the scientific community, but also industry and business, private landowners, developers, and the American public in general. "Contrary to the charges made by some 'fearmongers' on the Hill, NBS is non-advocacy and does not participate in regulatory decisions," he wrote.

But property rights advocates in Congress continue to make NBS a special target. The House committee added a provision to the appropriations bill that appears to prohibit agency scientists from conducting research on private land, even with the permission of the property owner.

Furthermore, a prohibition against the use of volunteer labour appears to have been extended to the use of data produced by volunteers. This would prevent the agency from using, for example, bird census data collected by the National Audubon Society and other similar groups.

If the budget cuts and restrictions survive the passage through the full House and Senate, they would "devastate the biological science capabilities of the Department of the Interior", said Pulliam. But all he can do now is hope for a saviour elsewhere in the House — or more likely in the Senate.

Time is running short. The full House is scheduled to take up the appropriations bill within the next two weeks, at the same time as Don Young (Republican, Alaska), an avowed ESA-hater, is planning to introduce his rewritten version of the Endangered Species Act. **Tony Reichhardt**

## 'All lines from space are engaged...'

**London.** The ubiquitous mobile telephone is threatening the future of radioastronomy, according to the European Science Foundation's Committee on Radio Astronomy Frequencies (CRAF).

Five major telecommunications companies are believed to be putting pressure on the International Telecommunications Union (ITU) in Geneva to delete a crucial phrase in the *Radio Regulations* that protects radioastronomers from interference on shared and adjacent frequency bands, according to CRAF's chairman-designate, James Cohen.

Radioastronomers share the 1610.6–1613.8 MHz frequency band with mobile satellite systems. A footnote forbidding "harmful interference" was inserted into the regulations at the request of British radioastronomers in 1992. But telecommunications organizations continue to disregard the clause, and five companies are now believed to have written to the US Federal Communications Commission asking for the footnote to be erased.

"Interference from radio and cell-phone

activity is a growing problem, which the ITU appears reluctant to recognise," says Cohen, co-author of a technical *Handbook on Radio Astronomy* published by CRAF later this month. "We face an uphill struggle."

Meanwhile the radioastronomy community, which occupies a minority position on the ITU, the United Nations agency that governs worldwide frequency allocations, is facing a new threat.

Telecommunications groups will ask the ITU at its next meeting in November to approve a network of low-Earth orbiting satellites to meet the growth in mobile communications.

The satellites will need access to several frequency bands that are crucial to radioastronomy. If the proposals are accepted, "up to half of all current radioastronomy activity is at risk," says Cohen.

Cable and Wireless, the global telecommunications group with interests in 25 countries, declined to comment on CRAF's charges. A spokesman added that no statement would be made until after the ITU meeting.

**Ehsan Masood**



# Budget axe threatens social science directorate at NSF

**Washington.** The social, behavioural and economic sciences (SBES) directorate of the US National Science Foundation (NSF) is widely expected to be eliminated after a powerful congressional committee instructed the agency to cut the number of directorates from seven to six.

The social sciences will still receive support from the NSF. Robert Walker (Republican, Pennsylvania), chairman of the House of Representatives Science Committee, said that the two-year NSF authorization bill that his committee passed last week "did not zero out" such funding, which came to a total of \$114 million this year.

But the order to shut a directorate will probably be applied to SBES, which is newer and far smaller than NSF's other six directorates. If that happens, the social sciences will have to compete with the 'hard' sciences for funds within other directorates, officials say — and social sciences are unlikely to do well in such a competition.

The directorate provides 60 per cent of all US federal support for research in the social sciences in US universities. Discussing social science research at a press conference in May, Walker said that the NSF had "wandered into these areas in recent years" when doing so was "politically correct". He added that NSF should, in his view, concentrate on the physical sciences.

The anticipated move to close the directorate is, therefore, something of a retreat by Walker, who had earlier hinted that the social sciences might lose their funding altogether. Neal Lane, director of the NSF, and Kumar Patel, president of the American Physical Society, were among those who had pressed Walker not to go that far.

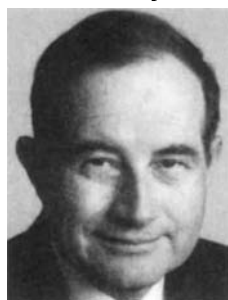
NSF will spend \$3.264 billion this year, and the bill passed by Walker's committee

last week gives it \$3.126 billion in the 1996 financial year, which starts in October, and \$3.171 billion in 1997. Most of the reduction, however, will fall on infrastructure funds; the amount of money for research grants would slip only slightly, from \$2.28 billion this year to \$2.226 billion in 1996, before recovering to \$2.28 billion in 1997.

Congress's budget process still has three months to run. But these NSF figures will not change much, as the House appropriations committees are likely to accept them, and the Senate's own proposals are broadly similar (see *Nature* 375, 168; 1995).

The figures mean that the agency, which funds most non-biomedical university research in the United States, is having a bad year by its own high standards, seeing its budget cut for the first time since 1986. But it is still doing far better than other research agencies, such as the Department of Energy and the National Aeronautical and Space Administration.

Warning voices such as those of George Brown, the top Democrat on the Science



**Lane: advised against eliminating funding.**

committee, and the American Association for the Advancement of Science, point out that the NSF faces real cuts of 20 per cent between now and the year 2002. But these are hypothetical projections built upon a number of political and economic imponderables, and should not be confused with the cuts of one third — or more — which threaten energy research programmes, for example, next year.

Walker's NSF authorization bill also instructs the NSF to block all grants to institutions that receive 'earmarked' funds from congressional committees for projects that have not been peer-reviewed. But NSF officials fear that this will require them to decide what is an earmark — angering universities and congressmen in the process.

Until a single NSF authorization bill is agreed by both House and Senate and passed into law, the agency has some discretion in how it complies with the provisions in the Walker bill. It will probably eliminate a directorate, for example, because it can keep Walker happy by doing so, without alienating any other powerful interests in Congress.

By contrast, the block on grants to certain congressmen's pet institutions would clearly alienate such interests, and the NSF is widely expected to ignore it. **Colin Macilwain**

# German council under fire over 'political' influence on decisions

**Bonn.** The Wissenschaftsrat, the science council responsible for assessing the work of research establishments in Germany, is itself to be evaluated. This follows a rare attack on its integrity in which the powerful state of Bavaria is claiming that the council has allowed itself to be influenced by the interests of federal politicians.

In response, officials of the Wissenschaftsrat claim Bavaria's accusations are based on self-interest. The state has been angered by a recent decision of the federal government to drop its full backing for the Wissenschaftsrat's proposals on university building and equipment, the costs of which are shared equally between the federal and *Länder* governments. This threatens Bavaria's ambitious university expansion plans.

This year, the federal government agreed to pay only DM1.8 million (US\$1.3 million) of its share, even though the Wissenschaftsrat had said that it should contribute DM2.3 million. In response, Bavaria said it wants to abandon the 25-year-old law on cost-splitting (see *Nature* 373, 95; 1995).

But its proposal received little support, as it would greatly disadvantage poorer *Länder*. As a result, Bavaria is now turning its attention to the science council's system of decision-making.

At the end of last month a meeting of the *Länder* prime ministers was asked to consider a proposal from Bavaria — which had already won the support of the *Länder* finance ministers — to limit the council's financing to two rather than five years while a review of its activities is carried out.

The prime ministers rejected Bavaria's proposal and renewed the Wissenschaftsrat's funding for a further five years. But they also agreed to a review of the council's activities in connection with university buildings and equipment.

A spokeswoman for the Bavarian research ministry says that Hans Zehetmair, the minister, suspects that the Wissenschaftsrat, which is supposed to make independent assessments of the needs of universities, has been influenced by pressures on the federal budget to limit its recommendations.

Zehetmair adds that the Wissenschaftsrat was due for a review, and that the university building issue provided an opportunity for it. But officials within the council believe his motive is to influence the debate on whether its formula for financing university building should be changed.

The organization of the review remains unclear. But research ministers will each nominate experts to the review panel — immediately raising questions about its objectivity, says Winfried Benz, general secretary of the council. **Alison Abbott**

## Mouse gene repository

**Munich.** The European Commission is expected to approve next week funding for a new European mouse gene repository, to be based at Monterotondo, near Rome.

A recommendation for such funding was approved last week by the consultative committee for the commission's biotechnology programme. The repository will contain a collection of mutant mouse strains to be made available on request to research scientists, and will complement a major genetics centre being established at Monterotondo (see *Nature* 374, 296; 1995).

A grant of ECU6 million (US\$4.6 million) from the commission over four years will also pay for the development of a supporting laboratory for the repository at Orléans in France. **A. A.**

# French science head faces cash crunch...

**Paris.** France's new government, which was formed in May following the election of Jacques Chirac as president, is expected to provide additional support for research on the human genome, ageing and AIDS. But overall funding for science is likely to be reduced as part of the government's drastic cuts in public spending.

The three research topics have been identified as priorities by Alain Juppé, the prime minister, in a letter to François Bayrou, the minister for national education, higher education, research and professional insertion.

Elisabeth Dufourcq, the secretary of state for research, last week described genome research as the "guarantee of French independence" in the "essential sector" of life sciences. Her comments follow warnings that French scientists risk being left behind without further substantial government support (see *Nature* 375, 175; 1995), and she backed proposals that have been made to establish a French megasequencing centre.

Dufourcq was speaking in Paris at her first press conference since her appointment. She has been in her job only a few weeks. But one look at the massive debts accumulated by the Centre National de la Recherche Scientifique (CNRS), and within the ministry's own research funds, has been enough to convince her that the most urgent task she faces is resolving the financial crisis in the research system. "I've become head of this ministry at a time when financial aspects are the priority", she said.

Dufourcq admitted being "very concerned" about the CNRS's financial situation; the agency faces an accumulated debt whose full size is estimated to exceed FF1 billion (US\$200 million), although the exact amount will not be known until an audit of its finances is completed later this month (see *Nature* 374, 586; 1995).

According to Dufourcq, her immediate priorities are to stabilize the financial situation facing French science, and prevent a recurrence of what she describes as a "dys-functioning" resulting from institutional, budgetary and management errors.

CNRS's debt has accumulated mainly because payments from the state have failed to keep up with the money promised by the government in the annual budget. Indeed, Dufourcq last week explicitly admitted that the state owes money to CNRS — an admission that the previous government had eschewed, but which has now been "welcomed" by CNRS itself.

Another cause of the agency's financial difficulties, claims Dufourcq, is that having based its recruitment on an overestimate of

the anticipated number of staff leaving, it now has about a hundred too many researchers. But with many CNRS researchers due to retire before 2005, a reduction in recruitment is not on the cards, she says, given that it would result in an unbalanced age distribution.



**Dufourcq: accepts promises to CNRS have not been met.**

Dufourcq did not say how she plans to tackle CNRS's financial difficulties. "At this time, we can only have a policy of least damage", she says. But according to one CNRS official, the most likely outcome is a rescue programme carried out over several years.

Indeed, there are unlikely to be any miracle solutions to the more general financial problems of French research. Juppé's government has set itself a priority of reducing general public spending in order to reduce the budget deficit and to finance its efforts to reduce unemployment.

Last week, for example, the government cut public spending plans for 1995 in a bid to reduce the anticipated budget deficit from FF371 billion to FF322 billion. The defence ministry, which had been allocated FF99 billion for running costs and FF102 billion for equipment this year, was worst hit, with its budget being cut by FF8.4 billion.

The budgets of other ministries will also

be cut, by FF5.2 billion. The government has not yet specified how much of this will fall on science, although Dufourcq says that research has not been "too badly treated, given the difficult budgetary situation". But she says that she intends to control research spending tightly "sector by sector", and to give priority to areas that could lead to "real advances" and not "dreams".

Otherwise, Dufourcq says she will mainly continue the policies initiated by Hubert Curien — who was science minister for much of the 1980s under the Socialist governments of François Mitterrand — and continued under Edouard Balladur.

In particular, Dufourcq says she would like to continue the system under which both universities and government-funded research organizations sign five-year contracts with the state fixing their main research objectives and the budgets needed to achieve them. But she emphasized that this policy needs to be pursued in tight collaboration with the heads of the individual research organizations.

Dufourcq's performance in the budget battles ahead is likely to be closely watched. Meanwhile, most observers in Paris say their attitude to her policies is one of "wait and see", giving her the benefit of the doubt, both while she finds her feet in the job, and during the honeymoon traditionally enjoyed by new governments formed after a presidential election.

**Declan Butler**

## ...and says blood claims are 'simplistic'

**Paris.** Elisabeth Dufourcq, France's secretary of state for research, last week expressed reservations about the handling of the contaminated-blood affair — one of the first political figures to do so.

In the early 1980s, haemophiliacs and other transfused patients were infected with HIV through the use of blood products contaminated with the virus. Dufourcq describes the current interpretation of the events as "oversimplified" and "completely reductionist".

Her comments coincided with the first skirmishes in France between supporters and opponents of the interpretation that alleges that officials deliberately ignored evidence that French products were contaminated to avoid importing safer alternatives, and delayed introduction of routine screening of donated blood for HIV until a French diagnostic test was available.

Dufourcq says the affair is not as straightforward as it has been painted. She describes it as a "collective error", in which much of the medical and scientific community must share responsibility for not recognizing the threat of AIDS sooner.

The affair, she adds, reveals the need to educate non-scientists "in the rudiments of statistics needed to exercise intelligent citizenship".

Indeed, writing in *Le Monde* in 1993, Dufourcq raised one aspect of the affair that has been neglected — the way in which politicians and scientists estimate risks in deciding to replace one health-care practice with another.

Both the courts and the media, for example, have argued that health officials should have been aware of the risks of contaminated blood, as a 1983 study of more than 2,000 haemophiliacs found that six showed suspected signs of AIDS. Dufourcq's arguments focus on why such findings are often ignored by scientists.

In particular, she attributes blame to the way that the significance of results is interpreted, claiming that too much emphasis is placed on broad trends, while rare results tend to be dismissed. While this approach usually serves science well, it can have "perverse effects", she argued, and "inhibit the most elementary common sense". **D. B.**



# Polish science 'in need of further reforms'

**Warsaw.** Widespread reforms in the organization of science in Poland, aimed at reducing rigidities in the post-Communist system and increasing incentives for researchers to improve the quality of their work, have been recommended by a panel of science policy experts put together by the Organization for Economic Cooperation and Development (OECD).

In particular, the panel proposes the creation of a fully fledged Ministry for Science, devolving funding responsibilities to a number of research councils, limiting the activities of the Polish Academy of Sciences, and increasing government spending on research and development to one per cent of gross national product (GNP) from its current level of about 0.7 per cent.

The panel also suggests that Poland should abolish the traditional system under which all permanent university staff are required to have a thesis-based second doctorate, known as a habilitated doctor (HD) degree. The "substantial opportunity costs" of this system, it says, are "a major barrier to Polish science becoming internationally competitive."

The OECD review was carried out at the request of the Polish authorities under a procedure that is widely used by the Paris-based agency. Speaking at a meeting in

Warsaw two weeks ago at which the panel's findings were discussed — and whose proceedings will themselves be published together with the report — Aleksander Luczak, Poland's vice-prime minister, said he welcomed the conclusions as "an independent and objective point of view" on Polish science.

The four-member panel gives its general approval to the main thrust of science policy reforms introduced since the end of the communist regime in 1990 (see *Nature* 372, 593–597; 1994). These have in particular included the creation of a State Committee for Scientific Research (KBN) to focus state support for science.

"[We] believe that the philosophy and competitive principles underlying the system are basically sound," it says, warning against "any moves to relax the existing centralized funding arrangements for science and technology which will



Home of the Polish Academy: abolition calls are 'unrealistic'.

be essential to achieving greater concentration and selectivity in Polish research".

But it argues for a clearer separation between responsibility for policy-making and for carrying out research. In this context, it suggests that the KBN should be relieved of its functions as a financing body — these would be passed to research councils and executive agencies — and that its role should evolve into "that of a true Ministry of Research and Technology", responsible for strategic policy decisions.

The panel acknowledges that the academy, based on the Soviet model, is widely seen as "part of the Communist legacy". But it adds that many powerful supporters argue "with some justification that its scientific track record is far superior to that of the universities in a number of areas".

Demands that it should be abolished, says that panel, are therefore "politically unreal-

## German research centres take on a historical name

**Bonn.** The umbrella group for Germany's 16 federally funded national research centres, which have recently been under pressure to increase their effectiveness, last week announced that it is to adopt a new name. It will also have a new central structure to enable it to play a stronger role in deciding the general strategy of the individual centres.

In line with the German tradition of naming research societies after famous scientists, such as Max Planck and Joseph von Fraunhofer, the body now known as the Arbeitsgemeinschaft der Grossforschungseinrichtungen (AGE, or association of national research centres) will in future be called after one of Germany's best-known scientists, Hermann von Helmholtz.

From November, the AGE will be known as the Hermann von Helmholtz-Gemeinschaft Deutscher Forschungszentren (HGF), the Helmholtz association of German research centres. The association's chairman, Joachim Treusch, hopes that the image of the nineteenth-century physiologist, physicist and mathematician will be more appealing than the association's present title.

"Our motivation is to signal to the outside world that the national research

centres do high quality research and have good collaboration with the whole of the research community," says Treusch.

A better image is certainly needed. The national research centres, whose activities range from research in nuclear safety to plasma physics, have been getting a bad press over the past few years, partly because of their alleged inflexibility — a consequence of the job security enjoyed by most of their scientists.

Criticism flourished in particular during the economic recession that followed reunification, as the centres are expensive to run. Their responsibility to carry out 'big science' or large interdisciplinary programmes means that they often have several thousand scientists.

The centres have recently been under political pressure to improve procedures for assessing the quality of their research, and their budget has not been increased for five years. At the same time, there has also been pressure from industry to make their research more directly relevant to industrial needs (see *Nature* 372, 4; 1994).

In response, research centres have cut staff numbers and those centres conducting a high level of applied research agreed last year to include more industrialists on their scientific and supervisory boards.

Behind the scenes, a more sophisticated defence was being planned. The new HGF will be more conscious of the need to let the public know exactly what it stands for: that is, says Treusch, long-term research programmes that are in Germany's general financial or social interests, and which require substantial resources and the willingness to take risks.

In addition, a new senate will be established to steer a coherent general research policy. Its members, who will be announced in November, will include eight *ex-officio* members representing research organizations, the ministries of research and finance, and the BLK, a body of politicians representing research interests of the federal and state governments.

A further 17 members will be elected from the scientific community, industry and elsewhere. The research centres themselves will not be represented. The head of the Helmholtz association will prepare and lead the senate meetings but will have no voting rights.

The senate will provide general advice on research strategy, oversee the mechanisms for ensuring the quality of research in the centres and promote cooperation both with other centres and with industry.

Alison Abbott

istic". But it argues that the academy, which is still responsible for the operation of a large number of research institutes, "should play a more limited and traditional role similar to that of the Royal Society or other major western academies of science".

The panel members say they decided to endorse requests "expressed by the majority of the scientific community" for a significant increase in government spending on research. They acknowledge the economic difficulties caused by the shift to a market economy. But they warn that, if the budgetary stringency resulting from the current transitional phase is prolonged, "Polish R&D may well lose its strongest assets".

Finally, the OECD panel is sharply critical of what it describes as the excessive "egalitarianism" that has come to dominate many universities since the fall of communism, arguing that it can lead to a fragmentation of programmes and a dispersal of resources that would be "particularly unfortunate" at a time of growing budgetary restrictions.

It argues, instead, for the concentration of a "substantial proportion" of both human and financial resources in centres of research excellence.

At a press conference held after the Warsaw meeting, Luczak, who took over as chairman of KBN at the beginning of this year, said that two of the OECD panel's most controversial sets of proposals were those on the future of the academy and on creating a centralized science funding agency comparable, for example, to the Centre Nationale de la Recherche Scientifique in France.

Equally controversial, he said, was the proposal to merge higher education institutions in various cities throughout Poland into "comprehensive universities". The panel suggested, for example, that the decision to integrate a medical academy into the Jagellonian University in Krakow could be copied in other cities.

Czeslaw Strumillo, vice-chairman of the KBN, said that implementing the proposals accepted by the government "would take time". Nevertheless, he described the report as a "rich source of information" that would be of considerable value to the government in formulating its future plans. □

## Strong yen prompts Japanese search for overseas talent

**Tokyo.** Under pressure from the rising value of the yen and Japan's prolonged recession, many Japanese companies are reorganizing their research and development (R&D) efforts both domestically and overseas.

This restructuring appears to be aimed not only at the conventional goal of bringing new products more quickly to market but also at finding ways of tapping into the creative talents of Western researchers to make up for their apparent absence in Japan in areas such as software design.

A survey last month by the *Nikkei Shimbun*, Japan's leading financial newspaper, of 35 major companies — including electronics, steel, automobile and pharmaceutical manufacturers — revealed that nearly two-thirds have reorganized their R&D divisions over the past year or will soon do so.

In addition, more than a third of those covered by the survey reported that they intend to set up new R&D facilities overseas soon or to strengthen existing ones.

For example, at the end of June, Mitsubishi Electric reorganized its R&D so that near-market research formerly carried out by various corporate research laboratories will now be done by the business section most closely related to the research.

Specifically, three laboratories, the Computer and Information Systems Laboratory, the Communications Systems Laboratory and the Image Systems Laboratory will be split and merged into two development centres, for information and communication systems and visual information systems, under the corresponding business units.

Eight other laboratories, including the company's central research laboratory near Osaka, will be merged into two centres, the Advanced Technology R&D Center and the Information Technology R&D Center.

Similarly, Matsushita Electric last year merged four research laboratories, including its central research laboratory, into a corporate headquarters for research while another five have been combined into a separate

headquarters for product development. This followed a reorganization by the rival company Sony in the previous year tying near-market research more directly to the 24 business groups that make up the company (*Nature*, 361, 193; 1993).

In all three cases, the goal of the restructuring has been to strengthen and speed up the introduction of new products to the market in order to help companies pull out of the recession. This contrasts with the so-called 'bubble economy' of the late 1980s, when Japanese companies poured money into new institutes carrying out basic research far from the market.

Such research is now being squeezed. None of the 35 companies surveyed said they expect to increase their support for basic research, although several also said that they do not plan any major reduction in such funding because of the long-term importance of such research.

Partly because of the rapidly rising value of the yen, several companies are planning to set up new research institutes in the United States and Europe. The average salary of a Japanese company researcher is equivalent to the most highly-paid researchers in the West, and Japanese companies are hoping to recruit talented Western researchers to the new laboratories.

But, unlike the institutes set up by Japanese companies in Europe and the United States in the late 1980s — many of which focus on quite basic research — the new facilities will be closer to market. One particular focus will be the development of new multimedia.

In August, Mitsubishi Electric will set up two R&D units in Europe. One in London will conduct research on digital broadcast technology, and will interact with another in France, focusing on telecommunications.

At the same time, two existing research groups in the United States — one working on high-definition television in New Jersey and another in California developing multimedia — will be brought under the wing of a single unit in Massachusetts, and their personnel will be expanded.

Japan's recent economic success has been built on combining high-quality production technology with mass production techniques. But many of the companies included in the survey said they think that future economic success will rest in the rapid development of software and new ideas — areas in which industrial research in Japan tends to be weak. So an additional incentive for establishing research facilities in the West is to gain access to what are widely seen as the more creative skills of Western researchers.

David Swinbanks

### D'Escatha to head French atomic agency

Paris. The French government last week nominated Yannick d'Escatha (right), currently deputy-director of the Atomic Energy Commission (CEA), to succeed Philippe Rouvillois as head of the agency.

D'Escatha, who is 47 years old and a graduate of the Ecole Polytechnique, outside Paris, joined CEA eight years



ago, with responsibility for activities relating to technology transfer.

After being appointed deputy-director in 1992, he supervised a comprehensive reorganization of the agency to focus its activities on long-term research in areas such as nuclear technology, reprocessing and safety

(see *Nature* 374, 104; 1995). □

# Trans-species transplants raise virus fears

**San Francisco.** With an experiment involving the transplant of baboon bone marrow into HIV patients in prospect, US federal regulators met last week to discuss the risks posed by xenotransplantation — the transplantation of organs or tissue from one species into another — and to collaborate on drawing up safety guidelines.

Government officials say that the potential risk of creating dangerous new viruses through cross-species transplants should be closely examined, and that their agencies can therefore legitimately claim authority over research in the field. They believe that special measures may be necessary, such as federal review of protocols, requirements for disease-free animals and long-term monitoring of transplant recipients.

Last week's three-day meeting in Bethesda, Maryland, was sponsored by the Institute of Medicine (IOM). Officials from the Food and Drug Administration (FDA) and the Centers for Disease Control (CDC) in Atlanta, Georgia, say that they plan to examine cross-species transplants of tissues and organs after enquiries from concerned institutional review boards, medical schools and the public.

In particular, both agencies have been spurred by a proposal from the University of Pittsburgh and the University of California, San Francisco (UCSF), to infuse baboon bone marrow into an HIV patient with the aim of boosting a weakened immune system with one that does not become infected with the virus.

Physicians at UCSF and Pittsburgh were ready to proceed with the experiment in April. But they were stopped by federal regulators, who wanted to examine the social and scientific implications of such work.

"We're not comfortable letting an experi-

ment like this go forward without some public discussion," says Philip Noguchi, head of the FDA's division of cell and gene therapy. "This is a very seminal case."

The experiment is at the forefront of what experts see as a new medical field with potential for explosive growth. They foresee a future in which baboons (because of their similarity to humans) and pigs (because of their abundance and organ size) would routinely provide bone marrow, hearts, lungs and kidneys for humans. The need is great: of those requiring a heart transplant, half die waiting for a suitable organ.

"The market size is only going to be limited by how good the technology is," says Alex Zisson of the investment analysts Hambrecht & Quist in New York. He estimates that within a few years, 50,000 pig hearts and 40,000 pig kidneys could be transplanted into humans each year.

But not everyone is enthusiastic. US regulators are concerned that a virus lying dormant in an animal could eventually become aggressive and deadly in a human environment. The research also raises issues about animal rights, as well as the morality of intermingling animal and human tissue.

Louisa Chapman, a medical epidemiologist at the CDC, claimed at last week's meeting that xenotransplants pose infectious risks to the general population, and should be carefully monitored.

She said that a growing body of data suggests that HIV itself may have originated from simian retroviruses introduced to human hosts, where they adapted and spread. She also cited cases such as the Ebola virus, which has emerged from an animal reservoir to kill almost 300 people in a recent outbreak in Zaïre.

More important, an animal virus could combine with a human virus, creating a more pathogenic and virulent microbe, Chapman says. "I cannot dismiss these concerns as mere science fiction."

The federal government acknowledges that the risks of xenotransplants have not been assessed. "We don't want to say we could have regulated this after another pandemic breaks out," says Noguchi. But he adds that in the HIV experiment — as with many other transplants — there are no other life-saving treatments, and that no cases of viral outbreaks from cross-species transplants have so far been documented.

Opponents of xenotransplants also include various animal-rights activists. James Walters, an ethicist from Loma Linda University who supports the HIV experiments, told the IOM meeting that xenotransplants raise a wider question of when it is ethical to kill an animal to save human life.

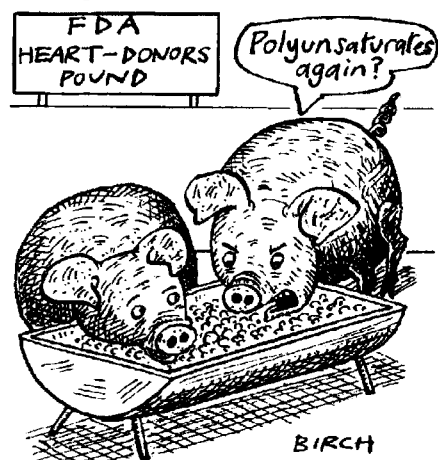
Baboons are not only close to humans in their genetic make-up but also have complex

social lives, Walters argued. "The greater a being approximates undisputed personhood — like you and me — the greater its claim to existence."

But such talk frustrates transplant patients, who are ready to organize against regulatory foot-dragging. "When people are dying, we need to show some urgency and passion," said Jeff Getty, an AIDS activist from San Francisco and a candidate for the baboon bone marrow experiment.

Getty and some researchers fear that a lengthy debate could lead to the suspension of work in cross-species transplantation just when it is showing promise.

Researchers also warn of the dangers of over-regulation. Suzanne Ildstad, who is



collaborating with UCSF on the AIDS experiment, accepted that the risks of a broad new technology should be considered.

But she expressed concern about the decision of regulatory agencies to become involved in small experiments such as her own. If the FDA had regulated human-to-human transplants during the early years, she said, officials would have stopped the experiments long before they were successful.

FDA and CDC officials plan to have guidelines ready for public comment by the end of the summer. They are preparing a draft for consideration by the FDA's Biological Response Modifiers Advisory Committee at a meeting next week which will also review the HIV/bone marrow protocol.

So far, committee members are moving toward some federal level review of xenotransplants, a registry of recipients, archiving of biological specimens and the quarantine of subjects for a short time where appropriate. The guidelines would also include provision for selection and screening of donor animals, regulators said.

Noguchi of the FDA refuses to predict whether the HIV experiment would win approval. But he said that this would not depend on completion of the guidelines.

**Sally Lehman**

## Markl to move to MPG

**Munich.** Hubert Markl, professor of zoology at the University of Konstanz and the first president of the Berlin-Brandenburg Academy of Sciences, has been nominated as the next president of the Max Planck Society (MPG). He will take over from the historian Hans Zacher, who completes his six-year term next June.

Markl, who is 57, was president from 1986 to 1991 of the Deutsche Forschungsgemeinschaft (DFG), which distributes most of the money for university research in Germany. His is the only name put forward for the MPG post, and a postal ballot is expected to confirm his appointment in August.

Meanwhile, Dieter Simon, director of the Max Planck Institute for European Legal History, takes over from Markl as president of the Berlin-Brandenburg Academy this autumn.

A. A.



# Wellcome and Zeneca form genetic alliance

**London.** In a novel three-way agreement, the Wellcome Trust and the British company Zeneca — formerly the pharmaceutical and life sciences division of ICI — have, together with the University of Oxford, announced plans for collaboration on research into multifactorial genetic diseases.

The three bodies have signed an agreement covering work on the genetics of rheumatoid and osteoarthritis at the Wellcome Trust Centre for Human Genetics. This was set up last year as part of the university's Nuffield departments of medicine and surgery, and brings together more than a hundred researchers at the university and the Oxford Radcliffe Hospitals Trust.

Under the terms of the agreement with what is widely seen as one of Britain's leading research centres into polygenic diseases, Zeneca will provide financial support for research at the centre, in return for which it will be granted options to exploit any drugs that emerge from the identification of relevant genetics targets.

The initial focus will be on bone disorders. But Graham Boulnois, director of biotechnology for Zeneca Pharmaceuticals, emphasizes that collaboration on further disease areas will be explored as opportunities arise. (Other diseases being studied by researchers at the centre include asthma, malaria and migraine.)

At the same time, officials at both the university and the centre claim that the agreement reached with Zeneca, which sets up a framework for close and continuous interaction between university scientists and those in the company, provides a model for potential collaboration with other pharmaceutical companies.

"We are not medicinal chemists, and are anxious to get ourselves locked in with partners that can help turn our discoveries into both diagnostic and therapeutic products," says John Bell, Nuffield professor of medicine at the university and one of the co-founders of the centre.

The three-way agreement, although not unique at a time of increasing collaboration between universities, private research foundations and private companies, nevertheless represents relatively new ground for each of the three partners.

For Zeneca, for example, which has long enjoyed close relationships with researchers in British universities, it is the first major external collaboration to be announced in genetics-related research, and follows the creation of a human genome group within the company last year.

Unlike rival UK pharmaceutical companies such as Glaxo Wellcome, Pfizer and SmithKline Beecham, each of which has announced collaborative deals with small genome-related biotechnology companies, Zeneca has until now been relatively slow in

building up such strategic alliances in genetics, although it has well-established links to British universities in more traditional areas.

Boulnois stresses, however, that the agreement with the Wellcome centre is just the first step. "Our intention is that it will be one of a number of interactions that are likely to include other academic centres worldwide, and could include other small biotechnology companies as well," he says.

Wellcome officials hope that the deal will help to answer critics who have been arguing that, up to now, the trust has lacked a strong strategy for ensuring that the results of the

of negotiating licensing arrangements through the university's own technology transfer company, ISIS Innovation.

University officials, however, say they are well pleased with the outcome of the negotiations. "In the past, medical charities which have funded work have not appeared to welcome licensing arrangements with industry", says June Clarke, director of research services for the university. "This new agreement is the way forward."

The agreement is not without its sceptics. Some, for example, question whether researchers at the Wellcome centre, which remains primarily devoted to fundamental research, will adjust easily to the pressures of a commercial agreement in which payment will be linked to the successful achievement of formal milestones — the standard practice with biotechnology companies.

But Zeneca officials say that they have been impressed by the attitude of Bell and his colleagues. "They are a highly entrepreneurial group," says Boulnois. "We feel that we have a very good arrangement."

Indeed, Boulnois says that, although the initial agreement with the centre is for a limited period — neither the precise time scale, nor the size of the company's financial commitment is being made public — the link with the centre could, depending on the successful achievement of plans already outlined, "go on for a very long time".

Bell has equal confidence in the arrangement with Zeneca, which he claims is "the only [British] pharmaceutical company with a serious human genome programme". He adds: "The whole deal has been very satisfactory from everyone's point of view".

Bell says that he is now keen to see similar deals negotiated with other companies, several of which — such as Roche and Merck — have already expressing interest in exploring potential links to research teams at the centre.

**David Dickson**

IMAGE  
UNAMIGABLE  
FOR COPYRIGHT  
FOR EASY  
REASONS

Zeneca Pharmaceuticals

**Follow through: Zeneca scientists will help turn genetic discoveries into products.**

research it supports — particularly in the field of human genetics — find their way into the health-care system.

Finally, the decision to negotiate a deal directly with Zeneca and Wellcome — under which the intellectual property rights will be held by the trust and licensed to the company, with royalty payments being shared with the university — represents something of a departure from what has up to now been the university's preferred route

## AAAS to send forensic experts to Haiti

Washington. Forensic anthropologists and other volunteers from Argentina, Guatemala and the United States will visit the troubled island of Haiti in September to exhume bodies in search of evidence of human rights violations.

The specialist team is being sent by the science and human rights programme of the American Association for the Advancement of Science (AAAS) at the invitation of a commission established by President Jean-Bertrand Aristide to investigate human rights abuses on the island between 1991 and 1994.

The team will be led by Clyde Snow, a leading US forensic scientist who is at present working on the investigation into

the Oklahoma bombing. It will spend four weeks on the island recovering bodies from two mass graves, at Fort Dimanche prison and near the capital, Port au Prince, and assessing their cause of death.

Other team members will help the commission — which is due to report by the end of the year — to establish a computer database to help it track connections between up to 20,000 human rights allegations which it expects to receive from the Haitian public.

Dan Salcedo, who is managing the programme at the AAAS, says that experts involved in two earlier programmes in Argentina and Guatemala will join the Haiti mission.

C. M.

# A philosopher's point of view

SIR — I should like to comment, from a philosophical rather than a scientific point of view, on John Godfrey's Commentary<sup>1</sup> and the correspondence that has followed it.

A recurrent problem in discussing these questions comes from the relationship between the terms 'person' and 'human being'. An individual who is entitled to certain kinds of protection, such as not being killed or experimented on without consent, can be said to have the status of a person. Creatures who have this status also, typically, have certain characteristics such as capacities for language use, reason, taking part in what are indeed called personal relations and so on. But a given individual does not have actually to display all, or perhaps any, of those characteristics in order to enjoy the status. If that were so, infants and the mentally incapable would enjoy no protection. Nor can one say that any individual who enjoys the status must have the potentiality for developing the characteristics: that excludes the irreversibly mentally incapable. Rather, you have to say that the status belongs to the kind of individual that typically displays the characteristics; and that, in terrestrial experience at least, is a human being.

So, in discussing origins, we can leave the term 'person' out of it — not because it has nothing to do with the moral argument, but because it represents the conclusion of the argument (protection should be given) and not the basis of that conclusion. The question must be entirely about the development of a human being, and in particular whether the zygote is a human being. One of your Roman Catholic correspondents<sup>2</sup> wrote that "as with any other animal species, fertilization is the moment when an individual human starts its development". This is in one sense indisputable: fertilization is the moment when something starts to develop into a human being. But this does not mean that it is the moment when an already existent human being starts doing something, namely developing. The reference to other species is well taken. By this argument, there would be no reason to deny that a caterpillar was a butterfly; but those who came to see butterflies, and put value on their beauty, would be reasonably disappointed if they saw only caterpillars.

The development of a human being is development *into* a human being, and it is indeed a gradual process. To prevent out-

rages such as experiment on neonates, we draw a line to govern these things, and we are wise to draw it early. But there is no reason in the metaphysics of persons or human beings that requires us to draw it at the very beginning.

**Bernard Williams**

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SIR — The question of what counts as a human being is more complex than Benjamin Libet<sup>3</sup> allows. The distinction he makes between "human life" and "the life of a human person" should be replaced by a threefold division.

Something is a mere bit of human life if it is both human and alive: a leukocyte, for example. A human embryo may not be a human being, but it is certainly a human entity in a stronger sense than this — as it is *both* human and alive and has a natural continuity with such as you or me. It is not at all surprising that some people will say that it is a human being — but no one would say such a thing about a blood corpuscle. But there are arguments against regarding a zygote as a human being<sup>4</sup>. And indeed it is somewhat unnatural to suppose so: one is inclined to take it for granted that every human being must have a head and a certain number of organs, and that no human being is spherical. If one could take a drug that would encourage uniovular twinning, no one would think it murder. A human bodily organization would seem to be necessary, like that established in the womb when an embryo turns into a fetus — an only roughly determinable time, needless to say, but a time noted by embryologists with no particular axe to grind. (The fact that the significant change is not instantaneous is of little importance.) An Aristotelian, for whom the soul was the "form" of the human being, might also take this view. A human being, one might say, has a ground plan. Of course some bits of the basic structure can be missing — one can be defective in various ways. But too many lacks destroy one's essence, so to speak. A car can lack a wheel, a seat, an engine, a bonnet. Take away too many such items and what is left is not a car, it is not even a defective one. In considering whether something is a human being we must not of course insist that the entity in question is fully developed, otherwise none of us would count until sexual maturity and the fusion of our long-bones. The word "person", where it does not mean "human being", or "man, woman or child", is nowadays best avoided: for some years now it has been defined by philosophers *ad hoc*, within a certain range, to suit their polemical purposes.

Further emphasis on the human brain

would be questionable. In particular, it would be a mistake to consider whether the entity had present thoughts, for we do not suddenly cease to be human beings when we are knocked unconscious. In discussing these questions, it helps to realize that we are dealing with the identity of a mammal, and that our question could be asked in all its essentials about canine pregnancy and puppy dogs. And it helps too to forget, if only for a while, about the abortion issue. Early abortion could be wrong even if it were too early to count as murder. On the other hand, there are plenty of philosophers around who suppose it can be all right to kill *born* children, as indeed was thought in the Roman republic.

**Christopher Coope**

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SIR — Libet<sup>3</sup> presents a hypothesis on what we might call the biological markers associated with personhood. But Libet's assertions — that the central role of the brain is constant in determining personhood, and that postnatal brain death can be equated with neural activity in the embryo/fetus — appear to be based on questionable assumptions.

It is agreed that postnatal brain death denotes the end of the individual's life and personhood. What is not clear is the relevance of this to the start of a life or personhood. A human's life and personhood are linear processes of development, and as such the inception of the process need not resemble the end of the process. Indeed, for most linear processes, the starting and ending characteristics are not the same.

Also unclear is the substantive relevance of comparing neural activity in a normal embryo/fetus with brain death in a postnate. The former is an entirely normal state, providing all the neural activity required at that stage of human development. The latter represents a highly abnormal catastrophic loss of a requisite function. Further, a declaration of brain death requires that there should be no reasonable expectation of regaining brain activity. With the embryo/fetus there is every expectation that such activity will arise in a predictable time and manner.

History is filled with otherwise good people employing some set of characteristics (many based on 'real' science) to explain why another class of *Homo sapiens* is subhuman or otherwise undeserving of basic human rights. We study the lessons, and pride ourselves on condemning these episodes as acts of folly, arrogance and inhumanity. Yet in 1995, here we are again.

**Michael A. Zarowitz**

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1. Godfrey, J. *Nature* **373**, 100 (1995).

2. Montuenga, L. M. *Nature* **374**, 10 (1995).

3. Libet, B. *Nature* **375**, 100 (1995).

4. Anscombe, G. E. M. in *Philosophy and Practice*, (ed. A. Phillips Griffiths) (Cambridge University Press, 1985).

## Correspondence

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

## Whale numbers

SIR — An advertisement placed by MJ Research Inc. in *Nature* for 20 April featured an item headed "Humpback whale still hunted, mtDNA shows". The paper referenced, by Baker and Palumbi, claimed only to have identified one sample that matched both Atlantic minke whale and humpback.

It is critical to Baker and Palumbi's conclusions that whale meat continues to be illegally imported into Japan that whale meat may be stored up to 10 years at  $-20^{\circ}\text{C}$ . The meat of all species referred to by Baker and Palumbi was legally imported into Japan until 1991.

The Baker and Palumbi paper has subsequently been seriously questioned at an international symposium on marine mammal genetics (La Jolla, September 1994). The assessment, by Nobuyuki Yagi of the Japanese Fisheries Agency, included the important observation (omitted by Baker and Palumbi) that a large number of toothed whales are legally sold in the Japanese market, yet none of these DNA type sequences was available to the investigators for comparison with the samples they analysed.

Potential contamination of the samples is also of scientific concern; the meat for analysis was obtained from retail stores and subjected to various processes (including marination) before being analysed. Such treatment may explain the mtDNA sequence identified as being midway between a minke and humpback, and another anomalous sequence claimed to be "intermediate between a sperm whale and a harbour porpoise". A more likely explanation for the latter sequence could be that 18 or more species of toothed whale (whose DNA profiles were unavailable to the researchers) were legally taken or stranded in Japan in 1993, and sold legally in food stores.

**Milton Freeman**

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## Site for disposal

SIR — There are a number of errors in the article "Nuclear storage decision postponed" (*Nature* 375, 174; 1995), not the least of which is the headline. It is almost inconceivable that anyone reading the Department of Environment announcement could conclude that it advocated postponement "until at least the latter half of the next century".

Instead, the government has said that Nirex should "proceed without any unnecessary delay, subject to meeting the necessary planning and regulatory

requirements". When announcing preliminary conclusions to the Review of Radioactive Waste Management Policy, Mr John Gummer, the Secretary of State for the Environment said: "The Review also looked at a fifty year delay and concluded that the balance of argument was against any such delay. The Government therefore believes that the repository should be constructed as soon as reasonably practicable, once a suitable site has been found."

The current target for first emplacement of waste is around 2010, which would imply that construction must begin in the first half of the decade. In fact, 2060 is the estimated date of repository closure, not the earliest date of the commencement of construction as stated in your article.

For the record, the current phase of the company's search for a deep disposal site commenced in 1987. Site investigations at Sellafield commenced in 1989. Nirex has so far spent some £300 million, much of this on scientific research, in its search for a suitable site.

**Tom Curtin**

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## Forced evolution

SIR — Your report on the Biotechnology and Biological Sciences Research Council workshop on forced evolution<sup>1</sup> does not mention whether Captain Lemuel Gulliver was a participant, nor whether the Grand Academy of Lagado was invited to send a representative.

Readers of *Gulliver's Travels*<sup>2</sup> will recall the genetic algorithm employed by the Professor in Speculative Learning and his 40 graduate students according to which, by the artificial selection of words randomly generated by tossing wooden blocks in a frame, "the most ignorant person may write books without the least assistance from genius or study".

"Six hours a day the young students were employed in this labour, and the professor showed me several volumes in large folio already collected, of broken sentences, which he intended to piece together, and out of those rich materials to give the world a complete body of all arts and sciences; which might however still be improved, and much expedited, if the public would raise a fund for making and employing five hundred such frames in Lagado, and oblige the managers to contribute in common their several collections."

It is not known whether the professor's grant application succeeded, but it is

known that Charles Darwin read *Gulliver's Travels* at Maer Hall between June and November 1840 (ref. 3).

**A. W. F. Edwards**

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1. Brookfield, J. F. Y. *Nature* 375, 449 (1995).
2. Swift, J. *Gulliver's Travels* (1726).
3. Burkhardt, F. & Smith, S. (eds) *The Correspondence of Charles Darwin* 4 (Cambridge Univ. Press, 1988).

## Authors scorned

SIR — Keith Frayn's objections to the unreasonable policies of certain scientific publishers (*Nature* 375, 100; 1995) are long overdue. As a research editorial assistant, I have seen numerous examples of everything Frayn describes, along with many examples of slipshod project planning. In one instance, it was more than two years before my boss received the page proofs of a book chapter he had been invited to submit. They arrived by regular mail — on a Friday — with an "urgent" memo stipulating that corrections be returned by overnight courier within 48 hours in order to avoid publication delays. Although we met this deadline, it was almost another year before the book was finally published. By that time, the entire volume was sorely out of date and virtually useless to readers intent upon keeping up with current research.

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## History of Xerox

SIR — In the leading article "Cutting off the sap to US research" (*Nature* 375, 263; 1995), it was stated that it would be impossible to trace the research and funding that led to the Xerox photocopier. Although the origin and development of the Xerox process is a mystery to almost everyone, it is known to a very few.

The process was invented by Chester Carlson, a graduate in the 1920s of the California Institute of Technology. The research leading to the present-day Xerox photocopier is as follows: research by Carlson, then Battelle Research Institute, then the Haloid Corporation which then transformed itself into the Xerox Corporation and refined the process.

It is ironic that Carlson, despite bestowing a great boon on society, remains almost completely unknown. He is the epitome of the Inventor Ignotus.

**George W. Housner**

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## Revolutionary birthdays

SIR — Michael Holmes (*Nature* 373, 468; 1995) points out that, among 19 physicists who took an early position on relativity and 28 biologists who took an early position on evolution, 80 per cent of the former and 92 per cent of the latter were born in the winter months (defined by him as including the period from October to April). Holmes assigns the difference to seasonal differences in nurturing. I assign it to Christmas.

For a child born during the summer months, the first Christmas is a phantasmagorical experience, with all those lights and sounds just when the senses begin to register. That experience is denied to children born during the winter months, either because it is too early for them to enjoy the proceedings or because they are born after Christmas. By the time the next Christmas comes along, they have already experienced one full summer, so the sights and sounds of Christmas lose much of their impact. As a result, summer-born children, impressed by Christmas, tend to grow up along more traditional lines and to be reluctant to embrace such devilish stratagems as relativity and evolution. In contrast, winter-born children, impressed more by nature with all its wonders and less by Christmas with all its dogmas, are more open to innovation, whether scientific or otherwise.

This conclusion is supported by the observation that 100 per cent of US presidents who held office in the eighteenth century were born in the winter months, compared to 66 per cent in the nineteenth century and 44 per cent in the twentieth century, paralleling the change of the United States from a revolutionary society to a bastion of traditional values.

All of the above is of course a paeon to the glory of small samples, which are wonderfully user-friendly in offering strong support to untenable theories.

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## Discriminatory Frontier policy

SIR — I wish to protest at the discriminatory policy of the Human Frontier Science Program (HFSP) regarding project funding for foreign nationals working in the programme's member states. In a recent full-page advertisement in *Nature* (4 May) the HFSP called for applications for grants "for basic research (up to 3 years) carried out jointly by research teams in

different countries. The principal applicant must be from one of the eligible countries." This statement is explicitly clarified in the HFSP's *Guidebook and Application Form for Research Grants*. Being from one of the eligible countries "... is defined as being a national (not a permanent resident) of one of these countries ...".

This definition is needlessly restrictive and is contrary to the tradition of free movement of research scientists. Although I work in Sweden, am employed by the Swedish Medical Research Council, receive funding from Swedish bodies, educate Swedish students and pay Swedish taxes, I am nevertheless ineligible to apply for an HFSP research grant as "principal applicant" because of my Australian citizenship.

The objectives of the HFSP emphasize the international nature of the programme and its support for younger researchers. But young researchers are notoriously mobile. Thus it would seem more in the spirit of these objectives if the HFSP eased its citizenship restriction for principal investigators. There must be many other researchers in HFSP member states, particularly the United States, who find this restriction unfair.

**Michael Lardelli**

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## Science in verse

SIR — Skill with words is not confined to those educated in departments of English, and the resources of metre, rhyme and its related tricks are open to everyone. There seems to be no reason to suppose that the propositional space, the set of all possible sentences in a language, needs to be divided between science and sacrosanct poetry, as I. A. Richards once famously proposed. Instead we can say that there is a continuum between more and less rigorous statements, with the looser, funnier or more pompous end taken by verse which normally, but perhaps not necessarily, maximizes its formal elegance and humour or dignity at the cost of its rational strength.

The literary world still jealously guards its vatic prerogatives, but the numbers of readers and the standards of reading diminish year by year, which is hardly surprising given the minimal intellectual and hence imaginative stimulation of the product. Is poetry, then, dead? By no means, it is only a rhetorical technique and may be transferred to any group of thinkers that chooses to take it up. I am convinced that this transition is already

taking place, and that there is a great deal of verse being generated by scientific workers and those interested in their ideas.

Your readers are invited to submit material or make recommendations for an anthology of verse and poetry related to science to be edited by Professor Masashi Suzuki of Kyoto University and myself.

Parodies, occasional *vers de laboratoire*, anonymous limericks culled from benches, and bitter couplets scribbled on conference programmes are as acceptable as deep night thoughts on the self and its genome, or the place of the carbon atom in our mysterious Universe. Mnemonics connected with any scientific matter would be most welcome.

Submissions should be sent to me by post, fax or e-mail. Texts may be sent on disk, or as attachments to e-mail, in the form of text files or as files compatible with the following Macintosh word processors: Microsoft Word, WordPerfect, WriteNow or Nisus Writer.

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## Street names

SIR — Street names are too mundane a matter for *Nature*, but they perhaps reflect science policy and/or demeanour.

W. F. Bynum begins his review of *Private Science of Louis Pasteur* (*Nature* 375, 25; 1995): "...when French schoolchildren were asked which historical figure has done most for France, 48 per cent named Louis Pasteur.... In no other country would such a poll have thrust a scientist in pole position."

In France, there is no city worth mentioning lacking a street, boulevard or place "Pasteur" — not to mention Berthollet, Monge, Cassini, Lavoisier, Laplace and so on. Italy names streets after Galvani and Volta. Such countries also name streets after artists and other eminent people.

Does any English city have a Newton or Darwin street or — perhaps with the exception of Stratford-on-Avon — a Shakespeare Square? Where is Turner or Gainsborough Road in London?

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### Correspondence

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

# Genetic information and life insurance

Robert J. Pokorski

**Genetic testing will become the future standard of medical care. Life insurers will also need access to genetic information if the insurance industry is to survive intact and if cover is to remain affordable.**

LIFE insurers are beginning to realize that genetic information is relevant to their assessments of mortality risk. If people use genetic tests to decide when to buy insurance and how much to buy, then insurers will also want to know the results of these tests.

But genetic tests may be difficult to interpret because of complex interactions between genes and the environment and variations in the age at onset and severity of disease. Doctors fear that patients will postpone genetic testing and thus miss the benefits of disease prevention and early diagnosis. There are also worries that insurers might use genetic information to invade privacy, discriminate against people with genetic diseases or deny insurance cover if genetic tests give unfavourable results.

My intention here is to show that genetics will become such an integral part of medical care that it will be impossible to draw meaningful distinctions between genetic and nongenetic information. Further, I argue that denying life insurers access to genetic information could lead to a fundamental restructuring of the life insurance industry and a drastic reduction in the number of people who could afford such cover.

## Genetic tests

The US Institute of Medicine's Committee on Assessing Genetic Risks predicts that "multiplex genetic testing" will soon become routine clinical practice, with many genetic tests done on a sample of blood or other tissue. The committee foresees a time when the public will be offered genetic screening by walk-in testing and mail-order and home-test kits<sup>1</sup>.

The ability to monitor genetic evolution from health to disease will make obsolete the traditional pathological distinctions between metaplasia, dysplasia and cancer. Molecular biological tests will replace standard histological techniques. To a life insurance company, it makes no difference if a person has a pathological diagnosis of moderate-to-severe dysplasia or its genetic counterpart. In either case, there is a high risk of cancer developing in the near future.

The PCR (polymerase chain reaction) test has been used to detect exfoliated neoplastic cells in the stool of patients with colorectal neoplasms, in the sputum of patients with lung and head-and-neck cancer, in the blood, bile and stool of

patients with pancreatic cancer and in the urine of patients with bladder cancer<sup>2,3</sup>. It will soon be common medical practice to use this DNA technology to diagnose genetic and nongenetic disorders in people who have developed a disease but are without symptoms. Distinctions between genetic and nongenetic information become even more blurred for tests that measure gene products<sup>1,4</sup>, including tumour markers such as prostate-specific antigen, and many common blood chemistry and haematological tests.

It is worth emphasizing here the distinction between predisposition and presymptomatic. If a genetic test identifies a predisposition to a disease, the disorder may or may not occur. By contrast a genetic test that detects a presymptomatic disorder identifies a condition that is already present but whose symptoms have not yet developed.

Many new forms of therapy will be based on genetic principles. As Henry Miller of the Institute for International Studies at Stanford University in California writes: "Somatic-cell human gene therapy applied to genetic defects, cancer, and cardiovascular disease may approximate in the first half of the next century what antibiotics have been in the second half of this century"<sup>5</sup>. Genetic testing will also be important in predicting the outcome of disease. For example, allelic loss from the long arm of chromosome 18 in patients with TNM stage II or III colorectal carcinoma has remarkable prognostic value<sup>6</sup>.

A list of common diseases with a genetic component now includes virtually every condition encountered in an average medical practice<sup>7</sup>. Information about family history, physical examinations and past treatment may also be seen as genetic<sup>1</sup>. Indeed, a report published in 1993 by the US National Center for Human Genome Research states that "[f]or policy purposes, it will become increasingly difficult to distinguish genetic from nongenetic diseases, and genetic information from non-genetic information" and goes on to observe that "[r]ecognizing that our genes affect many common diseases not previously thought of as genetic will transform the scope and meaning of terms such as genetic information, genetic test, asymptomatic condition, presymptomatic condition, and genetic predisposition to disease"<sup>8</sup>. Can insurers really be asked to ignore all this information

because it is genetic in nature?

A fundamental principle of private life insurance is equity. Policy-holders with the same or similar risk of death are charged the same premium. By contrast, public life-insurance schemes operate on the principle of equality: everyone at a given level of income pays the same amount, subject to laws governing maximum age for contributions.

## Risk classification

When people apply for life insurance, they are classified according to their expected mortality. The primary basis of risk classification is age. Yet in each age group the probability of death is greater for some people than others. Because of broad variation in life expectancy, all individuals in an age group cannot be offered private insurance on the same terms, so insurers use different mortality classifications. Most applicants worldwide are offered insurance at standard (average) rates, 2 to 3 per cent are charged a higher premium and 1 to 2 per cent are refused cover. (Despite the introduction of innumerable screening, diagnostic and therapeutic technologies over the past century, the percentage of people who have been able to obtain life insurance has in fact remained stable or increased.)

A private insurance system must retain the ability to identify the risks it is asked to insure (genetic or nongenetic), classify them into groups with similar expectations of loss and charge a price that reflects the level of risk posed by the group. This conclusion is supported by theories of private life insurance that have evolved over the past century, historical experience of assessment societies that collapsed because of failure to coordinate risk and premium, and basic economic principles.

Life insurance companies use both loss analysis and risk analysis when calculating premiums and determining the type of information needed to classify risks. Loss analysis looks at the past, and risk analysis looks at the present and into the future. Because the risk portfolios of all insurers already contain many policy-holders with genetic diseases, insurers would not be concerned if future purchasing decisions were based on previous buying practices, because these claims would be anticipated by loss analysis. This will not be the case once genetic testing becomes common medical practice, any more than it was with past medical advances that provided

patients and insurance applicants with better information about their health and expected longevity. People will use genetic information to guide their insurance purchases. Life insurance companies will need access to the same information to classify the risks they are asked to accept in order to coordinate risk and premium.

Suggesting that genetic information should not be used to classify risks is tantamount to advocating a fundamental restructuring of the life insurance industry. The economic scope of such an undertaking would be immense. Large-scale cross-subsidies would be required between healthy and unhealthy policyholders. Premiums would become intolerably high for most people. To retain some semblance of today's private life insurance mechanism, mandatory participation by all consumers would be required and the government would need to subsidize life insurance purchases of those unable to afford higher premiums.

Consumers would respond to a price increase in several ways. Some would buy less life insurance than if prices had remained the same, a reflection of the fact that insurance is a normal good, in an economic sense<sup>9</sup>. Others would decide to do without any protection because it would have become too expensive. Lower-income families would probably be the most adversely affected: life insurance is viewed as one of the few available investment options for low-asset households<sup>10</sup>. Some people might buy the same amount of life insurance but have less money for mortgage payments, financial investments or insurance for medical expenses, disability income or long-term care. And people in the group with the highest mortality risk would probably buy much more life insurance than before. They have a remarkable economic incentive: a high claim expectation and low premium rate.

It would be impossible to predict the overall effect. But it seems safe to say that the percentage of people who could buy life insurance would not surpass today's high levels; lower-income applicants would be disproportionately affected; the total amount of life insurance purchased would decrease<sup>11</sup>; and policyholders would have less money to spend on other things, as all economic costs are eventually paid for by individuals<sup>12</sup>.

Life insurance is inherently a 'discriminatory' product because premiums are based on differences in expected mortality rates. The differences are not intended to jeopardize basic rights but are necessary for actuaries to determine how much premium to collect to pay for future claims.

There is consensus in most countries that life insurers should not use race, ethnic background or religious preference in risk classification. Insurers have fully supported this position. But genetic information is different. Most genetic defects that

cause disease in later life are probably not inherited but are acquired as somatic mutations (mutations acquired later in life through exposure to environmental carcinogens or errors in DNA replication or repair). There would be nothing unique about charging a higher insurance premium if cancer had developed because of genetic injury caused by cigarette smoke, ultraviolet light or some other environmental toxin. The same could be said for presymptomatic cancer tests of urine, stool or blood specimens, as most cancer-causing mutations are somatic<sup>13</sup>.

Further, in the average patient (or insurance applicant) it will usually be impossible to determine if genetics or the environment dominated the disease process. There would also be unresolvable dilemmas in risk classification if genetic information were treated similarly to race, ethnic background or religious preference. If genetic tests were used to monitor the development of cancer, would applicants be treated differently if the cancer-causing gene was inherited rather than the result of a somatic mutation? When genetic factors are identified that correlate with increased longevity<sup>14</sup> or a lower risk of disease, will applicants with these favourable traits be told that this information cannot be used by insurers? Would a 30-year-old man with a life expectancy of only 10 years be treated differently if genetic rather than environmental factors were responsible?

Fault or control is not a concern of life insurers, only relative mortality risk. For example, many insurance companies use cardiovascular risk factors to estimate the chance of premature coronary heart disease. If there were several unfavourable risk factors, the company's medical director would never consider whether the applicant was at fault or lacked control because of failure to exercise, maintain a normal weight or follow a healthy diet.

Practical distinctions between fault and control will lose much of their meaning as more genes are discovered. Genes can influence cardiovascular risk factors such as obesity<sup>15</sup>, hypertension<sup>16</sup>, hyperlipidaemia<sup>17</sup>, diabetes mellitus<sup>18</sup> and family history<sup>1</sup>. Because nicotine is clearly addictive<sup>19</sup>, it may be only a matter of time before a genetic predisposition to the addictive effects of tobacco is discovered. Genes even play a role in alcohol and drug addiction<sup>20</sup>, two diseases often cited as examples of fault or lack of control. Insurers could not use fault or control in risk classification, nor could they restrict underwriting to situations where the applicant was thought to be at fault or in control. Most diseases are not a person's fault or under one's control. Exempting genetic diseases or information from risk classification would be equivalent to saying that applicants with all other disorders were responsible for their illnesses.

Genetic tests will be used to diagnose diseases that have already developed; most of these disorders (cancer and many chronic disorders for example) are not traditionally considered to be genetic in nature. In this instance, the tests can be viewed dispassionately as the latest in a series of new medical technologies intended to improve patient care. Concern about the use of genetic tests by life insurers is mostly irrelevant as the disorders — genetic or nongenetic, overt or presymptomatic — are already present.

Other genetic tests screen for diseases that might develop in the future. These would be used by life insurers in a similar way to tests already used in the underwriting process. For example, individuals with normal or moderately increased serum cholesterol levels are offered insurance at average premium rates; some applicants with significantly increased levels are charged an extra premium; and a small percentage are refused cover because of markedly increased levels. Extra premiums are required only rarely even though abnormal cholesterol tests are common: underwriting is not based simply on an isolated cholesterol value but also on factors such as age, type of insurance and other cardiovascular risk factors.

Life insurers are genuinely interested in finding ways to use genetic information without jeopardizing the ability to obtain insurance. The risk classification system is not perfect but it does allow the vast majority of applicants to buy cover. □

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# Making sense of dwarf-star evolution

**A remarkable double white-dwarf star appears to confirm a mechanism by which binary stars lose angular momentum but suggests that the pair may yet spring to life a billion years from now.**

IMAGES of planetary nebulae are among the most spectacular that telescopes can provide. There is a ring of glowing gas and, with luck, a small bright star at the centre. The ring of gas is not just a ring, of course, but a spherical shell that appears as a ring simply because the optical depth of a relatively thin shell is greatest at its extremities. 'Limb-brightening' is the technical term.

But how does a shell of glowing gas come to surround a star in such a way? The standard explanation is that planetary nebulae are produced late in the evolution of stars like the Sun. When the hydrogen fuel at the centre has been consumed to the tune of about 12%, the textbooks say, power production in the central core declines, the density at the centre increases and helium-burning begins in an even hotter inner core enclosed by a region still burning hydrogen. In other words, the power output of the star increases, its outer envelope expands under the influence of centrifugal radiation pressure and the star becomes a red giant, one whose luminosity is increased but whose external temperature is decreased.

That is only the first of many cataclysms. Soon, by the yardsticks of stellar evolution, even the helium core is diluted by the products of its own thermonuclear fusion ( $^{12}\text{C}$  and  $^{16}\text{O}$  for example) to such a degree that specific power production begins to decline again. There may well be a hiatus during which the vastly expanded envelope of the star can no longer be sustained by radiation pressure, so that it begins to collapse, while the core begins producing thermonuclear energy by the C-N-O process, turning lightish nuclei such as those into  $^{32}\text{Si}$  and eventually into  $^{64}\text{Fe}$ .

The explanation for the cataclysms that occur in stars in this condition is simple: the evolution of the core must eventually become much more rapid than the response of the envelope can possibly be. So there will be a time when a collapsing envelope encounters an almost suddenly increased flux of outward radiation. The most likely outcome of that collision of contradictory events is that a shell of the red giant's outer envelope will be blown away.

But will it glow as a planetary nebula? That depends on what kind of object is left behind. There will be a star of some kind where the thermonuclear core used to be. Whether the blown-off shell keeps glowing depends on the surface temperature of the remnant star. If that should be, say, 50,000 K, the star will radiate in the ultraviolet and

will excite even exotic spectral lines in the blown-off outer envelope. Then, indeed, the shell will glow. The remnant star will be a white dwarf, consisting mostly of helium at great density, say  $10^9 \text{ kg m}^{-3}$  or  $1 \text{ tonne cm}^{-3}$ , under which conditions the material is degenerate in the sense that its equation of state will be determined by quantum mechanics. Heat will be lost only slowly from such a star, chiefly because the surface area is only small.

Comparisons with supernovae are not irrelevant. They are also caused by the explosion of a star near the end of its tether. Again there is an expanding shell of glowing gas. The Crab nebula shows spec-

whose mass is small, perhaps less than half the solar mass. The difficulty is that progenitor stars smaller than the Sun will evolve more slowly, and that the Galaxy is probably not old enough for very small white dwarfs to have been formed by the standard mechanism. Is there some other way in which stars at the ends of their lives can shed substantial amounts of mass? That is the declared reason why T. R. Marsh from the University of Southampton has been looking for binary systems among the catalogues of white dwarfs (*Mon. Not. R. Astr. Soc.* **275**, L1-L5; 1995). And he has been lucky, finding a binary system in which each component is a white dwarf and in which there is clear evidence of motion in a tight orbit with a period of 3.47 hours.

Marsh's fellow-observers will no doubt be impressed by this demonstration of how it has been possible to extract from spectra taken with the William Herschel telescope on La Palma clear evidence that the H- $\alpha$  line from the star is split by the to-and-fro motion of the two stars in their orbit about their common centre. But the interest of the study is that it points to the way in which the double white dwarf may have come into being — and to what may happen to it.

Binary systems in general involve stars that differ from each other in mass or evolutionary state or both. That means that one will tend to accrete material from the other. But there are limits to the rate at which a star can accrete material, determined chiefly by considerations of angular momentum. The result is that material from the star losing material will spill over from within the region of tidal influence of the two stars; for a time they will each revolve about their common centre within this common envelope, but then the envelope will be expelled, carrying with it a substantial part of the angular momentum of the system, causing an abrupt shrinking of the orbit. In Marsh's view, this double white dwarf may already have suffered more than one of these episodes.

And what will happen to the system? The orbit is now compact enough to be a substantial source of gravitational radiation. In other words, even without a further common-envelope incident, the orbit will continue to shrink, so that the two white dwarfs merge with each other in roughly  $2.5 \times 10^9$  years. And then the result may be a star that burns helium for a time. For white dwarfs, it seems, there may be life after death.

**John Maddox**

## IMAGE UNAMBIGUOUS FOR A COPYRIGHT REASON REASONS

NASA/ESA

**The planetary nebula Hen 1357. This image from the Hubble Space Telescope reveals an equatorial girdle of dense matter and polar holes where bubbles of matter have burst.**

tacularly how the remnant star may be a neutron star emitting recognizable radio pulses. But the original mass of a star destined to become a supernova is great enough to sustain the process of nucleosynthesis beyond the nuclei in the middle reaches of the periodic table, with the consequence that even the heaviest nuclei are synthesized.

But supernova events are more spectacular than the throwing-off of planetary nebulae by several orders of magnitude. The surface temperature of the remnant neutron star, for example, will usually exceed  $10^6 \text{ K}$ . By the same test, the expanding shell moves outwards much faster than the glowing shell of a planetary nebula, whose velocity need not be much greater than the escape velocity from the surface of a red giant, itself much less than the escape velocity from the surface of a Sun-sized precursor.

The simple view of how white dwarfs come into being does not, however, account for the large proportion of them



# Faecal pellets in world events

Malcolm Walter

THE Proterozoic aeon — the interval between 2,500 and 540 million years ago, still occasionally lumped with the Archaean as the Precambrian — is yielding its secrets at an ever-increasing pace. Some time-worn perceptions have crumbled, perhaps none more spectacularly than the notion that all Archaean and Proterozoic sedimentary rocks are thermally metamorphosed. Few compounds in rocks are more sensitive indicators of heating than hydrocarbons, and we now know of many occurrences of Proterozoic hydrocarbon that would not have survived heating above 150 °C or so. On page 53 of this issue<sup>1</sup>, Logan and colleagues report a distinctive pattern of carbon isotope distribution in samples of such hydrocarbons. From this they derive a model for the geochemistry of the oceans of the time, one with far-reaching implications.

Discoveries like these have been made possible by developments in mass spectrometry that allow minute quantities of hydrocarbons to be detected and analysed, and contaminants (such as younger migrating oil) to be distinguished from indigenous components. The chemical complexity of natural mixtures of mobile hydrocarbons (bitumen, oil and gas), and of the immobile organic matter locked in sedimentary rocks (kerogen), is a rich fount of information. Oil explorers use this information to determine the thermal history of sedimentary basins and the source of any discovered oil. Tracing sources involves biomarkers, individual distinctive hydrocarbons, the ultimate origin of which can be traced to components of living organisms, such as light-harvesting pigments or the sterols of cell walls<sup>2</sup>. The palaeobiology of many sedimentary rocks is manifest not only as morphological fossils but also as suites of hydrocarbon compounds.

The hydrocarbon record is also being exploited for the information it contains on past environmental conditions. In the Mesozoic, for instance, contrasting carbon isotopic compositions of different hydrocarbon compounds can be used as a palaeobarometer for CO<sub>2</sub>, thus providing insights on the history of the greenhouse effect.

In the modern world, and throughout the Phanerozoic, biogenic lipids are depleted in <sup>13</sup>C with respect to bulk organic matter (both living biomass and kerogen in rocks), a consequence of the biochemical pathways of carbon incorporation into living organisms. Logan *et al.* have found a reversal of this relationship in Proterozoic sedimentary rocks. This is unlikely to be a result of different biochemistries in organisms of that time — all modern organisms

have comparable patterns of carbon isotope fractionation, and the main producers of organic matter in the Proterozoic are recognizable as modern groups such as algae and cyanobacteria. The authors therefore argue that it must be an environmental effect, and focus on the processes controlling the oxidation of organic matter as it settles through the oceans.

For all of Earth history before the latest Proterozoic, this 'snow' of dead planktonic bacteria and microalgae would have taken a long time to settle. As it sank it would have been decomposed by aerobic and anaerobic processes, which would have depleted the oceans in oxygen and, Logan *et al.* contend, also in sulphate (through the actions of sulphate-reducing bacteria). The result would have been stagnant oceans with low levels of dissolved oxygen and reduced concentrations of sulphate at depth. Then, some time very late in the Proterozoic, metazoans evolved muscular, unidirectional guts, and henceforth much of the organic matter in the oceans was packaged into faecal pellets, as in the modern oceans. So rather than slowly settling like fine snow, the organic matter now sank rapidly, more like hail. Consequently, it took its reducing power to the ocean floors and allowed oxygen to build up in the overlying waters. Thus Logan *et al.* claim to have recognized a revolutionary reorganization of geochemical cycles in the latest Proterozoic, induced by the evolution and proliferation of triploblastic coelomates.

Their hydrocarbon data indicate that this event happened between the times of the Rodda beds and Pertatataka Formation on the one hand, and the Oulburra Formation and Observatory Hill beds on the other, all in the Centralian Superbasin of Australia. The latter units are Early Cambrian in age, and the others are Terminal Proterozoic and, by correlation with dated units elsewhere, probably about 570–590 million years old<sup>3</sup>.

Logan and colleagues' hypothesis has many consequences. It predicts major differences in the distribution of organic matter between sedimentary facies either side of the advent of the gut proper. The Proterozoic carbonate lysocline may have been shallower than at later times. The patterns of fractionation of sulphur and carbon isotopes in the oceans would have been different, and indeed the known isotopic record seems to support major aspects of the hypothesis.

One particularly interesting consequence is the implied dating of a major evolutionary event, the origin of metazoans with muscular, unidirectional guts (effectively triploblastic coelomates).

According to Logan and colleagues, the geochemical evidence suggests that this happened sometime between about 540 and 590 million years ago. At first glance that seems to be consistent with the known distribution of the earliest animal fossils — the Ediacara fauna — which immediately predates the Cambrian and has an age range from about 545 to 565 million years ago.

There are, however, several reasons for considering that the Metazoa had a long, cryptic 'pre-history' (as the late Martin Glaessner called it). One interpretation of the decline in abundance and diversity of microbial structures, stromatolites, that began about 1,000 million years ago, attributes the decline to grazing and burrowing by metazoans; some interpretations of molecular phylogeny predict that the divergence of animal phyla began about that time; and simple, macroscopic medusoid fossils occur in rocks about 600–650 million years old<sup>4–6</sup>. Logan *et al.* could reasonably contend that this is consistent with their interpretation, because the earliest bilaterian metazoans probably were not coelomates. Nonetheless, there is a body of opinion (and a small amount of admittedly tenuous evidence) that there may well have been a metazoan fauna throughout the Neoproterozoic and that amongst that fauna there may have been microscopic coelomate bilaterians. This is an area that warrants much more research.

Logan and colleagues' own data also suggest an interpretation that does not involve any event in metazoan history. In their Fig. 2 (page 55) it can be seen that some Palaeozoic hydrocarbons have the carbon isotope distribution that the authors contend is characteristic of the Proterozoic. An alternative explanation is that the isotope pattern is characteristic of stagnant (anaerobic or dysaerobic) water bodies of any age, not only Proterozoic. Such stagnation may have been easier to achieve if, as is widely believed, there were lower levels of atmospheric oxygen during the Proterozoic, and so may have been more common then. This would not diminish the significance of the new data, but it would break the connection with metazoan history.

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The links between the hydrocarbon patterns and the palaeoenvironments of the sediments from which the samples were derived also merit scrutiny. If the different settling times between organic 'snow' and 'hail' are to be significant, water depths perhaps well in excess of storm wave-base would be required. The sedimentological data needed to assess this are not available for all the rock units involved, but it is notable that one of the units, the Bitter Springs Formation, is predominantly the deposit of a very shallow carbonate platform, with abundant evidence of exposure and non-marine deposition<sup>7</sup>. The samples used might well have come from evaporitic and siliciclastic beds that may be the deepest-water part of the formation, but it is unlikely that water

depths were over a few tens of metres.

It is possible to dispute aspects of Logan and colleagues' ideas because, as yet, the amount of available information is small. But at the very least they have developed one of those fruitful hypotheses that will have an immediate influence on research directions. Its diverse implications will lead to many tests, from further exploration of early metazoan history through to sedimentological studies and more work on the pattern of sulphur isotope distribution in Neoproterozoic (and younger) sedimentary rocks. Who can ask for more? □

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## APOPTOSIS

# The last cut is the deepest

Moira Whyte and Gerard Evan

ONLY a three-year holiday on the moon could excuse any biologist for not being aware that apoptosis, the innate programme by which cells commit suicide, is one of the hottest (and trendiest) pieces of modern biology. In the past few years, apoptosis has emerged from the backwaters of descriptive biology, in large part because it is now legitimized by the authority of both genetics and molecular biology. Indeed, apoptosis is now implicated in suppression of cancer and virus infection, and its inappropriate activation is blamed for many of humankind's most intractable pathologies — stroke, heart attack, Alzheimer's, AIDS and ageing. Now two papers, one by Nicholson and colleagues on page 37 of this issue<sup>1</sup> and the other by Tewari *et al.* in *Cell*<sup>2</sup>, identify a critical piece of the molecular jigsaw of cell suicide. Both demonstrate that a protease, an enzyme that cleaves other proteins, is a pivotal trigger, perhaps the pivotal trigger, of the cell suicide programme. This opens the way to possible pharmacological control of apoptosis, a pharmaceutical Holy Grail.

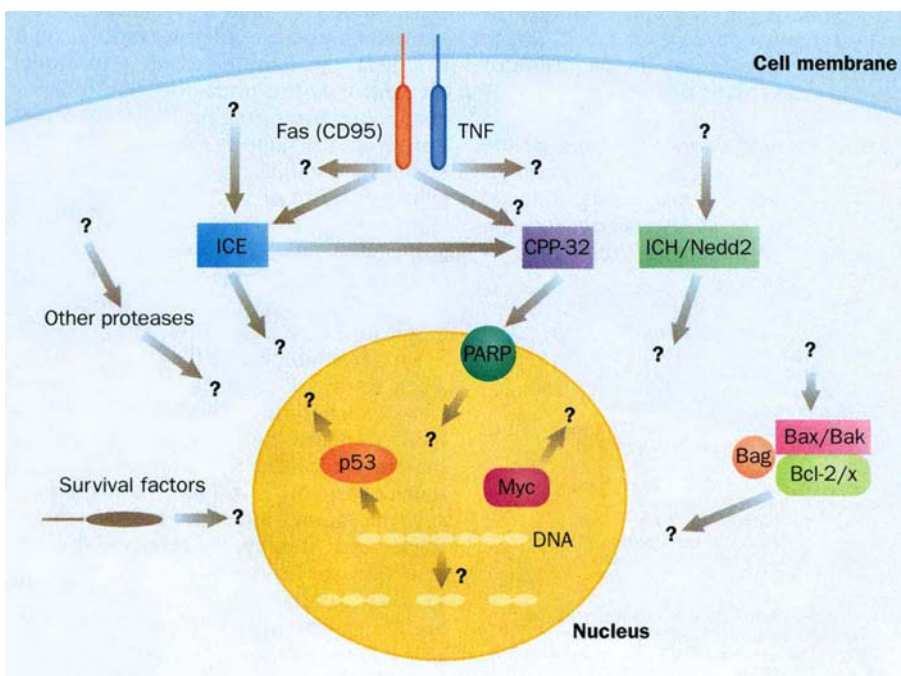
Much of our knowledge of the mechanisms of apoptosis comes from studies of the genetically tractable nematode worm *Caenorhabditis elegans*. During development of the hermaphrodite worm, specific cells undergo programmed suicide. This cell suicide is controlled by sets of genes, of which the principal protagonists are three — *ced-3* and *ced-4* are both required for death whereas *ced-9* blocks it. Last year, *ced-9* was shown to be homologous to a family of mammalian proteins of which the prototype is the *bcl-2* oncogene. Like *ced-9*, *bcl-2* blocks apoptosis, and it is this activity that is responsible for its oncogenic potential. The *ced-3* nematode

death gene also has mammalian counterparts. The first identified was the gene encoding the eponymous interleukin-1 $\beta$ -converting enzyme (ICE), a cysteine protease that activates pro-interleukin-1 $\beta$  by cleavage at two sites, cutting at aspartate residues at positions 27 and 116. ICE appears to be a principal intracellular target of CrmA, a pox virus protein that the virus presumably uses to suppress host-cell suicide and inflammation<sup>3</sup>. Functional homology between CED-3 and ICE is supported by the observation that ectopic expression of both CED-3 and ICE triggers apoptosis in rodent fibroblasts.

ICE is implicated in Fas-mediated

apoptosis<sup>4,5</sup> and in apoptosis following factor deprivation<sup>6</sup> or loss of contact with extracellular matrix<sup>7</sup>. However, there are two indications that ICE is not the universal trigger of mammalian apoptosis. First, both macrophage and thymocyte (other than Fas-induced) apoptosis proceed normally in ICE-deficient mice, despite major defects in their ICE-dependent generation of mature interleukin-1 $\beta$  and other cytokines<sup>5,8</sup>. Second, *in vitro* models of apoptosis, in which isolated nuclei undergo fragmentation typical of nuclei in intact apoptotic cells, implicate a protease with a different proteolytic specificity from ICE, namely prICE<sup>9</sup>. One target of prICE is the aspartate at position 216 in the enzyme poly(ADP-ribose) polymerase (PARP); PARP is involved in DNA repair and supervision of genome integrity. Nonetheless, as PARP-knockout mice develop normally<sup>10</sup>, PARP cannot be the only target for apoptosis triggered by CPP-32.

Both Nicholson *et al.*<sup>1</sup> and Tewari *et al.*<sup>2</sup> have now identified prICE as the ICE relative CPP-32, a cysteine protease that shares even closer homology with CED-3 than does ICE<sup>11</sup>. Like ICE, CPP-32 is synthesized as an inactive pro-enzyme that requires proteolytic activation. Unlike ICE, however, CPP-32 does not appear to undergo autocatalytic cleavage, so its activation presumably involves another aspartate-specific protease. This raises the possibility that CPP-32 is activated by ICE or by one of its multiple isoforms, by some other member of the ICE/CED-3 family such as ICH-1/Nedd2<sup>12,13</sup>, or possibly by the natural-killer-cell-granule protease fragmentin 2 and the cytotoxic-T-lymphocyte protease granzyme B (both of which share an



Factors that are known to affect apoptosis and the unknown relationships between them.

ICE-like predilection for cleavage after aspartate residues and trigger apoptosis). Although it is tempting to place CPP-32 near the apex of a proteolytic cascade leading to cell death, a strict hierarchy may not exist: rather, different family members may operate in different tissues or act on differing substrates within an individual cell to trigger apoptosis.

Of course, many questions remain. One concern is that the Yama/CPP-32 of Tewari *et al.*<sup>2</sup> shows far greater sensitivity to inhibition by CrmA than the apopain/CPP-32 activity of Nicholson *et al.*<sup>1</sup>. The reason for this discrepancy is unclear, but the possibility arises that the two activities are properties of different polypeptides. From a mechanistic stance, the most intriguing questions must be what does CPP-32 actually do to trigger apoptosis, what are its substrates and the consequences of their cleavage, and does active CPP-32 trigger a final and ineluctable process of apoptosis, or is reprieve still possible? On this last question, the jury is still out. The genetic interactions between the nematode *ced-9* 'anti-death' and *ced-3* 'death' genes (and, *mutatis mutandis*, *bcl-2* and CPP-32) provide no real information as to the biochemical relationship between these two proteins: Bcl-2 could act either upstream or downstream of CPP-32. Nonetheless, Bcl-2 expression does suppress ICE-induced apoptosis<sup>4</sup>, as do survival signals<sup>4,7,12</sup>, implying possible modulation of CPP-32 action.

Indeed, a bewildering diversity of factors influence apoptosis — not only cytokines and Bcl-2 family proteins, but extracellular matrices, oncogenes, tumour-suppressor genes, DNA-damaging agents, and elements of the cell-cycle machinery. Quite how all of these affect the actions of CPP-32 and related proteases remains part of a much larger mystery, as indicated by the profusion of question marks in the figure. □

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## Scales and stock markets

Allan Timmermann

DESPITE more than 30 years of research, no single characterization of the distribution of stock returns is widely accepted in economics. In their paper on page 46 of this issue<sup>1</sup>, Mantegna and Stanley propose a new and potentially powerful approach to model the finding that the distribution of high-frequency stock returns is highly peaked in the centre and has tails that are too 'fat' to be consistent with standard distributional assumptions.

Their idea is appealingly simple: use separate functions to model the centre and the wings of the distribution of returns on the Standard & Poor's 500 index. More specifically, Mantegna and Stanley suggest applying a Lévy stable distribution to the centre of the distribution and then adding exponentially declining tails. Stable distributions have the important property of additivity: if  $X$  and  $Y$  are drawn from a stable distribution, this will also hold for their sum. This is a convenient property for most economic data which are the result of time aggregation. Furthermore, the second moment of the Lévy stable distribution will only be finite for certain parameter values<sup>2</sup>.

Exponential decline of the tails guarantees that the variance of the distribution of stock returns is finite. Because one can never decide from a finite sample of observations whether the data are generated from a distribution with finite or infinite variance, the exponentially declining tails are best interpreted as an assumption based on empirical observations. Finite sample estimation of the tails of a distribution is complicated by the relatively small number of tail observations. So it is logical to attempt separately to model the centre of the return distribution, for instance by truncating the largest observations from the sample. Further research is needed to establish the point at which the centre distribution ceases to apply and the wing distribution takes over. Mantegna and Stanley suggest an 'eye-balling' procedure based on their Fig. 2 (page 48), but alternative estimation-based procedures might also be a possibility.

Proper modelling of the distribution of stock returns is important because of its significance for investors' management of risk. Standard financial models assume that investors are risk averse. Just how much risk an investor takes on by holding, say, the market index modelled by Mantegna and Stanley, is reflected by the distribution of those returns. Hence if an investor, wrongly, assumes that returns are drawn from a normal distribution, the risk taken on by holding the Standard & Poor's 500 index is likely to be much higher than expected.

The shorter the observation interval, the more do financial-returns data tend to deviate from the benchmark normal distribution: intra-day data is typically found to have a leptokurtic ('fat-tailed') distribution, whereas over longer periods, such as a quarter or a year, the leptokurtosis largely disappears. This information on the time-aggregation properties of stock returns is often ignored as researchers concentrate on deriving models for a specific period of time. In contrast, in their paper Mantegna and Stanley cleverly exploit the scaling properties implied by the Lévy stable distribution to estimate the parameters of the distribution of stock returns. Judged by their Fig. 1c (page 47), this scaling seems to be quite effective at least for the central part of the distribution.

The leptokurtosis observed in the returns of many financial indices is not necessarily best modelled as the outcome of draws from a single distribution. First, it is possible that returns could be generated by a mixture of distributions<sup>3,4</sup>. Discrete mixtures of normal or Student's  $t$ -distributions can generate fat tails while maintaining a relatively small value of the standard deviation of the resulting distribution. The mixing may also vary over time, as in the case of subordinated stochastic processes<sup>5</sup>, and reflect the difference between clock time and activity time, taking variations in trading volume as the proxy.

Non-stationarities<sup>6</sup> or time-varying conditional moments<sup>7</sup> of the process generating the returns data are other possibilities. Volatility of stock returns tends to cluster in time<sup>8</sup>, periods of highly variable stock prices being more likely to follow if prices previously were volatile than if they were calm. The large literature on ARCH processes documents this persistence in the volatility of returns and these models can generate fatter tails in the distribution of stock returns compared to the normal distribution. (ARCH, or autoregressive conditional heteroskedasticity models, allow the second conditional moment (variance) of a time series to be a function of the history of past realiza-

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tions of the process. GARCH, or generalized ARCH models, provide a more flexible way of accounting for the persistence often observed in squared stock returns.) Interestingly, Mantegna and Stanley report that the scaling property of stock returns in the sample is not well approximated by a GARCH(1,1) model. It is possible that this would not be so if a more elaborate ARCH model were used, but their result is consistent with frequent findings that ARCH models cannot fully account for the leptokurtosis observed in high-frequency financial returns.

Changes in institutions or in the economic regime may also account, at least partially, for the observed leptokurtosis in the distribution of stock returns. Because this view allows for the possibility that large outliers in the tail of the distribution

of stock returns are drawn from a different distribution than the observations in the centre, it falls well in line with the approach suggested by Mantegna and Stanley. No study so far has been able to explain the events during the market 'meltdown' of 19 October 1987 as a 'reasonable' draw from a distribution that also describes the price dynamics during more normal times. A class of Markov switching models<sup>9</sup> allows for time-dependence in the mixing of distributions of stock returns corresponding to different regimes. For common stock indexes there seem to be at least two regimes, one with high variance and low (negative) mean returns, and another with low variance and positive mean returns. These models can be combined with ARCH processes to provide a possibly better fit

of the distribution of returns<sup>10</sup>.

In view of the strong evidence of time-varying parameters of the distribution characterizing high-frequency stock returns, the scaling approach proposed by Mantegna and Stanley should not be regarded as a substitute for existing models such as ARCH or regime switching. Instead it is likely that the two approaches can be fruitfully combined by using the distribution suggested by Mantegna and Stanley to model the residuals from one of the classes of models which has proved successful in forecasting the volatility of stock returns. □

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## OBITUARY

### Christian B. Anfinsen (1916–95)

CHRIS Anfinsen died suddenly on 14 May at the age of 79. He was the quintessential protein chemist, acutely aware of both the chemical and biological sides of the field. In 1972, while he was chief of the laboratory of chemical biology at the National Institutes of Health, he shared the Nobel prize in chemistry with Stanford Moore and William Stein, awarded for pioneering work relating the structure and function of the enzyme ribonuclease. At the time of his death he was professor of biophysical chemistry at Johns Hopkins University in Baltimore.

In the late 1940s, before even the structure of DNA was known, Anfinsen began an experimental study of protein biosynthesis. The incredible complexity of this process was then only just becoming evident, but the general outline and basic requirements were eventually laid out in his seminal book *The Molecular Basis of Evolution* in 1959. His careful thinking in this uncharted area was to serve him well.

The appearance in the mid-1950s of a large sample of the bovine enzyme pancreatic ribonuclease, provided with some foresight by the Armour Company, enabled him to diversify his activities in a more chemical direction. Although he worked on aspects of the amino-acid sequence of the ribonuclease chain, the sequence itself was largely solved by Stein and Moore at the Rockefeller Institute: it was only the second complete sequence to be determined. Chris and his team focused on the effects on the enzyme's structure and catalytic properties of covalently modifying the peptide chain by proteolytic cleavage and by chemical means. The possibility for modifying a specific sequence genetically was of course still decades away.

One modification he was trying to

achieve was to reduce the four disulphide bonds in the native molecule to eight sulphhydryl groups. No method could be found by which this reaction could be carried to completion without the use of a denaturing solvent such as 8 M urea to open up the molecule and



make the S–S groups accessible to the reducing agent. This process resulted in the complete loss of the enzyme's catalytic activity, but it was not clear whether this was due to the denaturing conditions, which was to be expected, or whether the conversion of the four S–S groups to eight SH groups might itself be enough to remove the activity.

To prepare a linear chain for sequencing, Stein and Moore also needed to cleave the disulphide groups, and chose oxidation to yield eight sulphonic acid functions; this reaction is irreversible, so all catalytic activity was lost. To no-one's surprise, the product in aqueous solution showed no evidence of any residual

structure apart from a random coil.

In contrast to sulphonic acid groups, sulphhydryl groups can readily be reoxidized to the disulphide form. Anfinsen dialysed away the urea from his reduced sample in an oxygen-free atmosphere: nothing seemed to happen. The solution was then left in a beaker open to the air. Overnight, a large amount of the original catalytic activity of the enzyme was restored — the protein had reformed its native structure, unaided! The enormous implications of this seemingly simple experiment were immediately obvious to Anfinsen: all the information necessary to convert the randomly coiled peptide chain into its unique, biologically active structure was contained in the sequence of amino-acid residues in the chain. Herein lay the answer to the last step in protein biosynthesis — straight chemistry, no biological 'magic'.

Confirmed in later years by dozens of papers from his own laboratory and thousands from others, this statement is the central dogma of what is now termed the 'protein-folding problem'. A detailed understanding of this eludes us even now, but the central fact still stands. The fascinating recent work on chaperonins has generated a whole new level of complexity, but the information transfer from gene to native structure remains dependent only on the sequence. A much longer review would be required to cover all of Chris Anfinsen's contributions to the chemistry and biology of proteins, but he will retain his major place in the history of science because of a simple experiment involving a beaker and a prepared mind. Frederic M. Richards

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# Potassium tells a tale

Stuart Ross Taylor

In two papers in *Geochimica et Cosmochimica Acta*<sup>1,2</sup>, Munir Humayun and Robert Clayton report accurate measurements of the isotopic abundance of potassium in samples from various parts of the Solar System. These data place an important new constraint on the interpretation of early events during the formation of the system.

The composition of the primordial solar nebula, from which the Sun and planets formed, is well known. This is because the abundances of the non-volatile elements in the solar spectra, including that of iron<sup>3</sup>, match those measured in primitive carbonaceous meteorites. The components of the original nebula are usually divided into 'gas' (hydrogen, helium and the noble gases, making up 98%), 'ices' (water, ammonia and methane, about 1.5%) and 'rock' (about 0.5%). Indeed, the planets reflect this by their grouping into the gas giants (Jupiter and Saturn), the smaller ice giants (Uranus and Neptune), and the very small, rocky, inner or terrestrial planets (Mercury, Venus, Earth and Mars) that contain so little of the original nebula that they might be ignored to a first approximation, except that we are standing on one of them.

It might have been expected that the inner rocky planets would have similar compositions. What is surprising is that their compositions do not equate with that of the original rocky component of the nebula. Along with their marked deficiencies in the ices (the Earth has only about

500 parts per million water, less than one-thousandth of the original nebular budget), they are depleted in elements, for example the alkali elements, lead, bismuth, thallium and so on, that are volatile at temperatures below about 1,200 K.

This depletion is measured most readily by the relative abundances of a volatile element, such as potassium, to a refractory element such as uranium. Both ele-

"a factor of 3–4 better than the best published results"<sup>1</sup>. Previous measurements that have reported variations in this ratio are now shown to be in error, including some from Clayton's laboratory<sup>4</sup>.

Why potassium? It is one of the very few moderately volatile elements that meet the criteria of a multi-isotopic, low mass, lithophile element that is not subject to isotopic fractionation during geological processes. Although there has been a history of reported variations in potassium isotopic ratios in terrestrial samples, Humayun and Clayton were unable to find any, and the unexpected result is that

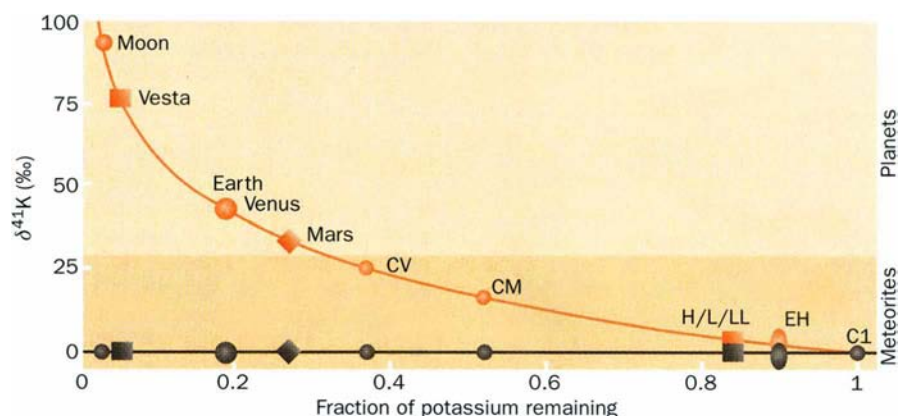


FIG. 2 Potassium isotopic compositions for planets and meteorites. The bottom line shows the values measured by Humayun and Clayton. The top curve shows the expected values calculated on the basis that the potassium loss was due to evaporation, starting from the C1 composition and assuming a Rayleigh distillation. Adapted from ref. 2.

ments are natural  $\gamma$ -ray emitters, and so can be measured remotely on planetary surfaces. And although they are dissimilar chemically, both are excluded from the most common mineral lattices, and so remain as a coherent pair through planetary differentiation processes. Thus the surface K/U ratio reflects their bulk planetary abundance.

From the extensive data now available, it is clear that the Earth, Venus, Mars, the Moon and some meteorites derived from the inner asteroid belt are strongly depleted in potassium (Fig. 1). Although this is a conveniently measured element, the deficiency extends to all volatile elements, with the more volatile elements showing greater depletions. The mechanism for this element loss has long been debated, and basically there are two views — that the volatile elements were evaporated from the individual bodies at some stage during formation, or that their loss occurred much earlier in the solar nebula by some different process.

Humayun and Clayton<sup>1,2</sup> have now answered one of these questions with their measurements of the isotopic abundance of potassium. They have surmounted considerable technical difficulties and have achieved a precision of  $\pm 0.5$  parts per thousand in measuring the  $^{41}\text{K}/^{39}\text{K}$  ratio,

the potassium isotopes are remarkably uniform, not only on the Earth, but throughout the inner Solar System. The only unambiguous example of isotopic fractionation of potassium is that shown by lunar soils. These surficial materials are exposed to volatilization by micro-meteorite impact in a hard vacuum on a small body. They have lost perhaps 15% of their potassium and show a loss of lighter  $^{39}\text{K}$  relative to  $^{41}\text{K}$ .

Apart from terrestrial samples, Humayun and Clayton have analysed chondritic and achondritic meteorites, a chondrule, two refractory calcium–aluminium inclusions and a variety of lunar rock samples. Although the potassium content of these samples varies by a factor of 30, they show no detectable isotopic variations. Figure 2 shows the experimental data (lower line), compared with the isotopic variations expected if the observed potassium depletions were the result of evaporative loss (upper curve).

The result is that attempts to account for the wide range in the abundance of potassium (and the other volatile elements) in the inner Solar System by evaporation from an originally homogeneous reservoir do not work. The primitive carbonaceous chondritic meteorites, with their volatile elements conveniently

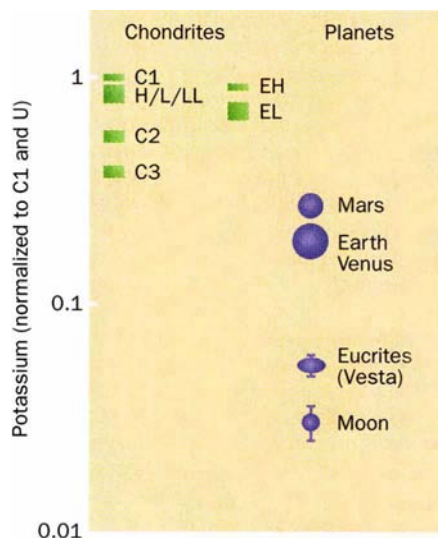


FIG. 1 The depletion of potassium in various chondritic meteorites (chondrites) and planets. The carbonaceous chondritic meteorites (C1) represent the original solar nebular value. Vesta is the asteroidal parent body for the basaltic eucritic meteorites. Adapted from ref. 2.

removed by vaporization, cannot be used as building blocks of the planets. So these papers provide no support for popular planetary building models<sup>5-7</sup>.

Incidentally, they also dispose of the old idea<sup>8</sup> that the depletion of sodium and potassium in lunar basalts was due to evaporation as the basaltic lavas were extruded on to the hard vacuum and low temperatures of the Moon's surface. The low abundance of the alkali elements in the Moon (less than 20% of the Earth's already depleted budget) is now shown to be an inherent property of the material that formed the Moon. Evaporative loss of potassium during the formation of chondrules and refractory inclusions is also ruled out, so that the depletion of volatile elements in chondrules can no longer be ascribed to loss during the flash-melting episode that melted the precursor silicate dust balls.

Chondrules are major components of the chondrites, dated at 4,563 million years old<sup>9</sup>; the refractory inclusions give slightly earlier ages of 4,566 million years<sup>9</sup>. So it seems that the depletion of volatile elements occurred before the formation of the chondrules, or the refractory inclusions, and thus before 4,566 million years ago. Because these are the earliest available samples of the Solar System that we can measure, we must retreat to a remoter epoch in our search for the mechanism for depletion.

Although Humayun and Clayton<sup>2</sup> incline to condensation from an initially hot nebula as the cause, considerable problems remain. The observed oxygen isotopic heterogeneity in meteorites seems unlikely to have survived in such a high-temperature environment, while condensation might be expected to affect the isotopic ratios. It is tempting to ascribe the depletion to early violent solar activity that swept water and other volatiles out to a 'snow line' at five astronomical units from the Sun, where condensation enabled the rapid, early growth of Jupiter. All of these problems provide rich pickings for further investigation.

About 20 years ago, oxygen isotope data from Clayton's laboratory<sup>10</sup> clarified the relationships among the meteorite

groups, indicating that they mostly came from distinct reservoirs, and swept away models that produced the various classes by derivation from primitive meteorites. The new data will likewise enable cosmochemists to concentrate on early solar nebular processes, and to assemble everything from meteorites to planets from materials that have already been depleted in volatile elements, without having to invoke evaporation and vaporization. The

## PATTERN RECOGNITION

# Time for a new neural code?

Terrence J. Sejnowski

THE relative timing between spikes from neurons in your two ears conveys information to your brain about the locations of sound sources in space. On page 33 of this issue, John Hopfield<sup>1</sup> suggests that the relative timing of spikes between cortical neurons conveys information about the location of individual components in the multidimensional space of a sensory stimulus such as a smell: recognition of patterns occurs when each spike arrives simultaneously at a 'recognition cell'.

Temporal codes are not new, and one of the simplest — synchronous firing — has already been suggested as a candidate for binding sensory features together in the cortex<sup>2</sup>. What is new is Hopfield's observation that if the logarithm of the analogue strength of a sensory cue is encoded by the time advance of a spike, then the information contained in the relative timing of spikes in a population of neurons can be transmitted rapidly, in a scale-invariant form, and decoded into a computationally versatile radial basis function representation<sup>3,4</sup> (see figure). However, this time-advance code requires a precision of spike initiation and a sensitivity to the temporal coincidence of spikes from different neurons that until recently was considered beyond the capability of cortical neurons<sup>5</sup>.

Sensory neurons in cerebral cortex respond vigorously when the right combination of sensory cues is present, but there is great variability in the timing of spikes from trial to trial. As a consequence, the average firing rate, which ignores spike timing, is believed to be the primary cortical information code. The apparently noisy properties of electrical signals in cortical neurons would seem to preclude a temporal code that depends on the timing of spikes<sup>6</sup>. But even if the firing rate is the primary information code, there is danger in ignoring spike timing entirely, because one man's noise can be another man's signal.

Indirect evidence that cortical circuits are capable of processing with high temporal precision has been lurking for many

work is a nice example of progress in science by the erection of testable hypotheses, while the small number of samples used in this decisive experiment recalls the comment of Charles Darwin that "six samples are enough for a scientist". □

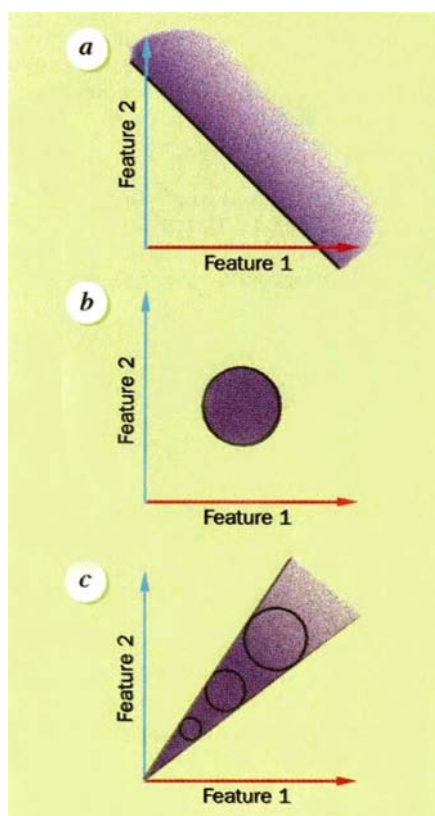
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years in the psychophysical and physiological background. When images are flashed on the retinae, onset-time differences in the range of a few tenths of a millisecond between the two eyes are interpreted as differences in depth<sup>7</sup>. As the primary visual cortex is the first area in the brain where information from the two eyes converges onto a single neuron, this suggests that timing information is preserved at least up to that point, and that precise temporal differences can influence perception. Measurements of orientation-tuned neurons in visual cortex indicate that the first few spikes in a response are as tuned as the average firing rate<sup>8</sup>: evidently, cortical circuits only need a few spikes to form a judgement about their preference. Finally, experiments on cortical neurons in a slice preparation have shown that the precision of spike timing in response to the repeated injection of the same 'noisy' current, meant to mimic synaptic bombardment *in vivo*, is less than a millisecond<sup>9</sup>. Although spike initiation in cortical neurons can be quite precise, cortical synapses do not appear to be as reliable, at least under conditions studied in slice preparations.

Hopfield provides strong theoretical arguments of why the cortex might use a time-advance code. Nearly all models of

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Comparison of different ways that cortical neurons could represent sensory features. A neuron achieves its peak firing rate when the value of two features (vertical and horizontal axes) are located in the shaded region. The neuron in *a* fires best when the values fall above the line; the neuron in *b* only responds well when the values lie within the circle. If the response is only sensitive to the ratio of the two values, as in *c*, then the region of maximal firing is a wedge. The time-advance coding scheme<sup>4</sup> provides a direct way to compute the receptive fields in *c*.

neural networks start with the assumption that the firing rate is a sigmoidal function of the summed inputs. This allows single neurons to make linear discriminations in the space of input features, as shown in *a* in the figure. A radial basis function, in contrast, only responds to inputs in compact regions of a feature space, as in *b* in the figure. The time advance coding scheme suggested by Hopfield can naturally compute such radial basis functions, if the inputs to the neuron have the appropriate time delays. In addition, the neuron would also respond vigorously if the values of all of its inputs were multiplicatively scaled by a constant factor (*c* in the figure), which would produce a constant time advance in the logarithmic coding scheme.

It would be impossible to determine by recording from a single neuron alone whether it was computing with time advances; only simultaneous recordings from several neurons could reveal how the information was propagated and decoded. In recordings from pairs of cortical

neurons, reliable differences in spiking times ranging from a fraction of a millisecond to over 100 milliseconds have been reported<sup>10,11</sup>. There is also evidence for time advances in the rat hippocampus, where single hippocampal neurons respond to spatial location: as a rat moves through the preferred place, the timing of the spikes relative to a background of 4–6 Hz theta rhythm changes from phase lag to phase lead<sup>12</sup>. Thus, the relative timing of spikes in the population could carry information about relative location.

The timing of spikes is already well established as a means for conveying information in the electrosensory system of electric fish<sup>13</sup>, in the auditory system of echolocating bats<sup>14</sup>, and in the visual system in flies<sup>15</sup>. Does the timing of spikes

mean anything in cerebral cortex? If so, then there must be a sophisticated system in the cortex to adjust the time delays of spikes. We need to design new experiments that manipulate spike timing in cortical neurons, perhaps by selectively interfering with specific timing mechanisms. Although the time-advance code is indeed a clever scheme, we must not forget that nature, which has been in the coding business for a long time, is cleverer than we are. □

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## CELL SIGNALLING

# A taste of things to come

Charles S. Zuker

TASTE transduction is one of the most sophisticated forms of chemotransduction<sup>1,2</sup>, operating throughout the animal kingdom from simple metazoans to the most complex of vertebrates. Its purpose is to provide a signalling response to non-volatile ligands (olfaction provides a highly specific, extremely sensitive response to volatile ligands). Higher organisms have four basic types of taste modality: salty, sour, sweet and bitter. Each of these is thought to be mediated by distinct signalling pathways that lead to receptor cell depolarization, release of neurotransmitter and synaptic activity<sup>3</sup>. Although much is known about the psychophysics and physiology of taste cell function<sup>4</sup>, very little is known about the molecules and pathways that mediate these sensory signalling responses. To complicate matters, some taste-receptor cells respond to more than one taste modality, raising important mechanistic questions about signal cross-talk and information encoding and decoding. Now, on pages 80 and 85 of this issue<sup>5,6</sup>, Margolske and colleagues offer us some surprising insights into these signalling cascades.

Sour and salty tastants are believed to modulate taste cell function by direct entry of  $H^+$  and  $Na^+$  ions through specialized membrane channels localized on the apical surface of the cell (reviewed in ref. 7). In the case of sour compounds, taste-cell depolarization is thought to result from  $H^+$  blockage of  $K^+$  channels, and salt transduction from the entry of  $Na^+$  through amiloride-sensitive  $Na^+$  channels. None of the molecular components of the sour or salty pathway has been identified.

Sweet and bitter transduction are believed to be G-protein-coupled transduc-

tion pathways. However, nothing is known about the membrane receptors activated by bitter and sweet compounds — this is surprising, given the tremendous biotechnological and pharmaceutical applications for bitter antagonists and sweet agonists. The transduction to sweet tastants appears to involve cyclic AMP as a second messenger<sup>8–10</sup>. The current view is that a seven-helix-transmembrane sweet receptor activates a  $G_s$  type G protein, which in turn activates adenylate cyclase. Increased cAMP leads to the activation of protein kinase A and the phosphorylation and blockage of a family of potassium channels. In this regard, cAMP has been shown to mimic the effect of sweeteners, leading to receptor-cell depolarization via a blockade of a potassium conductance. Interestingly, there is also evidence for an amiloride-sensitive conductance in sweet transduction<sup>11</sup>.

Bitter transduction appears to make use of a number of intracellular pathways relying on different signalling strategies. For instance, denatonium, one of the most bitter substances known to humans, leads to release of calcium from intracellular stores in some taste-receptor neurons<sup>12</sup>. This suggests an involvement for phosphoinositide-phospholipase C (PLC) signalling in this pathway. But cyclic nucleotide phosphodiesterases (PDE) have also been implicated in bitter transduction. The PLC pathway probably involves the modulation of  $Ca^{2+}$ -activated conductances, whereas the PDE pathway may alter the gating of cyclic-nucleotide-modulated ion channels.

The only molecule to be identified so far in the bitter signalling cascade is gustducin<sup>13</sup>, a  $G\alpha$  subunit that is highly specific to taste neurons and very similar



to visual transducin. Transducin is the  $G\alpha$  subunit in vertebrate photoreceptor cells and is responsible for coupling the light-receptor molecule rhodopsin to a photoreceptor-cell-specific cGMP phosphodiesterase. Active PDE hydrolyses cGMP into GMP and because the light-regulated channels are opened by cGMP, the transient reduction in cGMP causes the closing of these cation-selective channels. Thus, the absorption of a photon by the vertebrate photoreceptors causes a brief hyperpolarization of the cell.

In their papers<sup>5,6</sup>, Margolskee and colleagues report two intriguing findings on vertebrate taste receptor cells. The first, by Ruiz-Avila *et al.*<sup>5</sup>, demonstrates the presence of visual transducin in rat and bovine taste-receptor neurons. The authors describe studies involving immunohistochemistry and the polymerase chain reaction to show that transducin is expressed in some taste receptor cells; moreover, indirect assays provide hints that transducin functions in bitter taste transduction. These results are highly suggestive and will fuel research in this area, but one must be cautious about extrapolating expression studies into functional ones — particularly important here as gustducin and transducin share about 80 per cent amino-acid identity. The most rigorous experiment, namely the generation of transducin-knockout mice, is yet to be done, although gustducin knockouts have recently been produced (R. Margolskee *et al.*, unpublished results). The analysis of these animals as well as transducin/gustducin double mutants should provide important insight into the biology of these pathways. Interestingly, if transducin does indeed function in coupling a bitter receptor to phosphodiesterase, then it follows that the receptor in question is likely to share significant homology with rhodopsin.

In the second paper<sup>6</sup>, Kolesnikov and Margolskee describe the presence of a cyclic-nucleotide-suppressible conductance in frog taste-receptor neurons. This is something of a novel mechanism, as cyclic nucleotides have never been shown to suppress or inactivate ion channels

directly. This is in contrast to cyclic-nucleotide-gated ion channels in which direct binding by cAMP or cGMP opens the channels. Single-channel studies should provide a detailed description of the biophysical properties of these channels and the role of cyclic nucleotides in their regulation. Kolesnikov and Margolskee also show that the electrophysiological responses of some receptor neurons to the two sweeteners saccharin and NC-01 (assuming that both taste sweet to frogs) are modulated by IBMX, an inhibitor of phosphodiesterase, suggesting a role for

phosphodiesterase in their transduction pathway.

Given that most sweet transduction responses are believed to be mediated through activation of adenylate cyclase, the results presented in these two studies give us a taste of the complexity and excitement of this field. □

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## PLANET FORMATION

# Stellar gerontology

Steven V. W. Beckwith

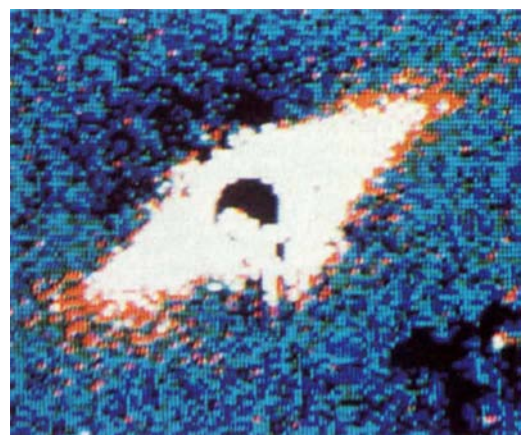
LIKE their well-preserved counterparts on the silver screen, stars appear to be ageless. Yet the inexact art of fixing stellar ages figures prominently in the study of all aspects of the evolution of the stars and their environs. Working with the refurbished Hubble Space Telescope, Lanz, Heap and Hubeny<sup>1</sup> have reassessed the age of the star  $\beta$  Pictoris, and conclude that it is either 12 million years (Myr) or more than 300 Myr, with the younger age preferred, as opposed to the 100–200 Myr previously assumed<sup>2</sup>.  $\beta$  Pictoris is our best candidate for a star with a mature planetary system<sup>3</sup>, yet the growth of large planets takes a few hundred Myr<sup>4,5</sup> — longer than the apparent time available.

Direct observations of extrasolar planets are currently beyond our means, but we can see the small particles inevitably accompanying planetary birth and ageing. In mature planetary systems, small particles are created as the by-product of collisions between asteroids and other large bodies. Collisions within the asteroid belt give rise to the zodiacal light seen in the Solar System and visible to the naked eye at dark sites just after sunset and before sunrise.

The total mass in zodiacal particles is a tiny fraction of that in planets, but the total surface area is much greater because of their small size. Viewed from the nearest stars, the zodiacal light would outshine the planets by several orders of magnitude over the entire visible and infrared spectrum. Thus, the first searches for planetary systems by intelligent beings on  $\alpha$  Centauri, say, would see the zodiacal radiation around the Sun long before Jupiter and the other planets could be detected.

The discovery of zodiacal-like particles around the stars  $\alpha$  Lyra (Vega),  $\beta$  Pictoris

and  $\alpha$  Piscis Austrinus (Fomalhaut), made during routine infrared observations of so-called 'standard stars' by the IRAS<sup>6,7</sup> craft, sent shock waves through the astronomical community. Here, for the first time, was good evidence that planetary systems did, indeed, exist outside of the Solar System. A striking image of the particles around  $\beta$  Pictoris<sup>8</sup>, confined to a disk seen edge on, was obtained soon after the initial discovery and was reproduced



J. Grady *et al.*, Univ. Hawaii/Science Photo Library

FIG. 1 Particles around  $\beta$  Pictoris, in the form of a dust disk here seen in white. The image was taken with the 2.2-m telescope at Mauna Kea, Hawaii.

in News and Views earlier this year (374, 762; 1995). A false-colour infrared image is shown in Fig. 1.

Particularly important was the realization that the particles could live no more than a few million years before spiralling into the stars from the force of stellar radiation (Poynting–Robertson drag); our zodiacal particles suffer the same fate and have similarly short lifetimes. The stellar ages were estimated at several hundred million years, meaning that the particles must be continuously replenished, presumably by collisions from larger bodies already in orbit about the stars.

But unlike the zodiacal particles, which

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are created just beyond the orbit of Mars at about 3 astronomical units (AU) and whose density increases towards the Sun, the particles around Vega,  $\beta$  Pictoris and Fomalhaut are created at nearly 100 AU, more than twice the orbit of Pluto, and there are holes in their distribution as they spiral in towards the stars. The central clearing inside a few tens of AU is most readily explained as the dynamical sweeping up of particles by larger bodies (presumably planets) in orbits similar to those of the Solar System.

The greater extent and mass of the

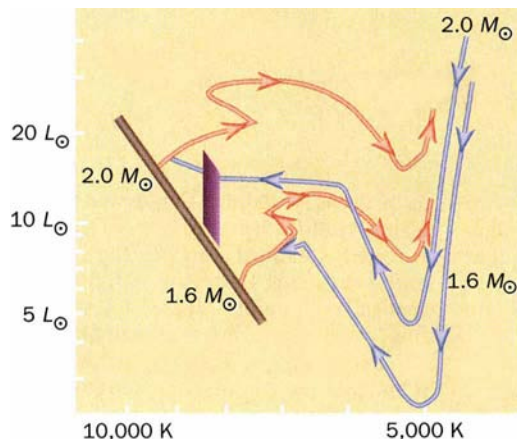


FIG. 2 The Hertzsprung–Russell diagram used by Lanz *et al.* to estimate the age of  $\beta$  Pictoris. The trapezoid is the error-box for the stellar location; the lines with arrows indicate the evolution of a star of a particular mass (1.6 or 2.0 solar masses,  $M_{\odot}$ ) over time. It takes approximately 20 Myr to evolve from the top right corner to the main sequence (grey line); a star spends approximately 300 Myr on the main sequence before evolving up and to the right in the diagram.

particles around the distant stars compared to the zodiacal particles suggested that planet formation is continuing, that is, that the largish bodies at many tens of AU still needed time to coalesce into giant planets. To be fair, we would not yet be able to see a similar mass of particles in the outer regions of the Solar System, but our prejudice is that planet formation ceased more than 4,000 Myr ago. Therefore, the age of these other systems is an important element in our assessment of the rate at which planets are built around other stars.

Estimating stellar age normally relies on a measurement of the luminosity and temperature of the surface compared to theoretical calculations of how these quantities change with time for a star of a given mass. Figure 2 shows the measured position of  $\beta$  Pictoris in the luminosity–effective temperature diagram (called a Hertzsprung–Russell diagram) together with evolutionary tracks calculated from stellar models. The trapezoidal region delineates Lanz and colleagues' best estimate for the position of the star after accounting for interstellar extinction. Unfortunately, the region is consistent either with a very young star, 12 Myr

old, just moving onto the grey line (the main sequence) or a relatively old star, more than 300 Myr old, just moving off the main sequence line after considerable evolution.

Unfortunately, the ambiguity of the age diminishes the significance of the new result. The authors favour the younger age simply because the particle disk around the star is much denser than any other examples detected to date. If  $\beta$  Pictoris is only 12 Myr old — the preferred solution — there has probably not been sufficient time to create giant planets such as Jupiter, but Earth-sized planets could have arisen. On the other hand, the transient particle lifetimes are easier to understand as the remnants of a much larger reservoir remaining from the initial collapse of the nebula during stellar birth. An older age for the star leaves open the question of why  $\beta$  Pictoris is unusual in its large particle density, although it would be easier to explain the particles as collisions from larger bodies in orbit. Clearing of the inner hole by smallish (Earth-sized) planets orbiting at a few tens of astronomical units could occur for either age.

Although useful, this new estimate of the age leaves open the issue of whether very large bodies orbit the star. Our conventional theories suggest a few large proto-planets can grow by runaway accretion in about 1 Myr (ref. 9). Subsequent growth of giant planets requires 10 to 100 times as long. If the young age for the star can be confirmed, it implies that the formation of giant planets has not taken place but is happening now. That holds out the tantalizing possibility of training future telescopes or interferometers on  $\beta$  Pictoris to witness the processes that assembled the giant planets in our own Solar System long before we were around to appreciate their significance. □

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## DAEDALUS

## Steam speaks!

DAEDALUS deplores the conventional loudspeaker. Its clumsy vibrating cone reproduces the high frequencies very crudely; and it is so small compared with bass wavelengths that it radiates them very badly. He now has a new idea.

He points out that the vapour pressure of any liquid rises with temperature, and very dramatically compared with sound pressures. He calculates that the vapour pressure change from heating water by a mere 0.01 °C could generate a thunderous 100-dB sound-pulse. A sheet of water could create deafeningly loud sound simply by minor fluctuations of surface warmth. But how to do it at the kilohertz rates required for sound reproduction?

Daedalus's cunning idea is to heat, not the whole mass of water, but merely its surface. Not only does this save power; the bulk of the water stays cold, and cools the surface instantly when that power is withdrawn. Both heating and cooling can thus be carried out very rapidly. He has two ideas for heating a water surface selectively. The first is to dope it with a detergent whose ions cluster at the surface. (This is the effect which stabilizes detergent foam.) Because water conducts electricity via dissolved ions, an audio signal passed through such a solution should selectively heat its surface. His other scheme exploits the 'skin effect' which limits high-frequency current to the surface of a conductor. By modulating an inaudibly high 'bias frequency' with the output from an audio amplifier, audio-frequency heating could again be confined to the water surface.

A tank of warm water, however, is an inconvenient loudspeaker. The commercial version will be a sort of vertically hanging wet blanket, with electrodes round the edge. It will be shrouded in a loose but sealed enclosure of sound-transparent plastic film, containing a small dehumidifier to condense evolved vapour and return it to the blanket.

Cheap, simple and with no moving parts, DREADCO's 'Steam Speaker' will transform sound reproduction. It will handle the loudest and most complex sound with authoritative high fidelity. A big enough version could match the longest sonic wavelength, and its acreage of surface could radiate kilowatts of pure thunderous bass. Even rock musicians will cringe at the power available to them. More thunderous still, its output could be focused by curving the wet blanket, or by using many Steam Speakers as a phased array. Assault, demolition, rain-making by vibrating the clouds and even anti-aircraft duties seem feasible.

David Jones

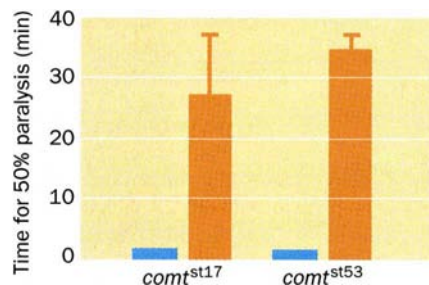
# A *Drosophila* NSF mutant

**SIR** — Recent work suggests that the molecular machinery mediating vesicle targeting and fusion is conserved in both constitutive and regulated secretion (reviewed in refs 1, 2). Here we report that the *Drosophila* mutant *comatose* (*comt*) is defective in one component of this conserved secretory apparatus, the *N*-ethylmaleimide-sensitive fusion protein (NSF). Furthermore, *comt* exhibits an apparent defect in synaptic transmission which is the first functional evidence that NSF is involved in this process.

NSF was initially identified as a protein required for vesicular transport in the constitutive secretory pathway present in all eukaryotic cells. The requirement for NSF in this process was shown to occur after vesicle formation and targeting, and thus NSF was proposed to participate in vesicle fusion, or a step closely preceding it. Subsequently, NSF was used to identify other proteins that function in vesicular transport, including the soluble NSF attachment proteins (SNAPs)<sup>3</sup> and the SNAP receptors (SNAREs)<sup>4</sup>. Because SNAREs were found to be identical to previously characterized synaptic proteins required for synaptic transmission, NSF was implicated in this process as well.

We recently initiated a genetic analysis of synaptic transmission in the fruit fly *Drosophila melanogaster* by identifying neurally expressed *Drosophila* homologues of NSF and the SNAPs<sup>5</sup>. The gene encoding the NSF homologue (*dNSF*) was localized to the 11 D9-E4 region of the X chromosome. Candidate *dNSF* mutations, uncovered by the same set of deletions that remove the *dNSF* gene, were then identified among existing recessive mutations. Mutations of the *comt* locus, identified by Siddiqi and Benzer on the basis of their temperature-sensitive paralytic phenotype<sup>6</sup>, were among the candidate mutations mapped to this region (K. S. Krishnan, personal communication).

To examine the possibility that *comt*



**FIG. 2** Analysis of NSF transformants in the rescue of the *comt* temperature-sensitive paralytic phenotype. Flies heterozygous for a P-element construct bearing a *dNSF* cDNA under the control of a heat-shock 70 promoter<sup>7</sup> were subjected to 38 °C heat shocks of 15 and 30 min duration on successive days to induce expression of *dNSF* protein. Flies were tested for paralysis at 38 °C 24–28 h after the second heat-shock treatment (as described in ref. 6), but with the following modification: filter paper inserts in the bottom of the tubes used in the water bath test allowed slightly impaired flies to right themselves. As a control, nontransformant *comt* siblings from the same crosses that generated the test flies were subjected to the same heat-shock regime and examined for temperature-sensitive paralysis in identical fashion.

mutations are alterations in *dNSF*, PCR (polymerase chain reaction)-derived complementary DNAs containing the entire *dNSF* open reading frame were obtained from two different *comt* alleles and wild-type flies, and sequenced. Both the *comt*<sup>st17</sup> and *comt*<sup>st53</sup> alleles contain a single missense mutation relative to the wild-type cDNA sequence (Fig. 1). Furthermore, both *comt* mutations are G-C to A-T transitions, the most common type of mutational change caused by ethylmethane sulphonate, the mutagen used to generate *comt* alleles.

As a more definitive test, transgenic flies were constructed to determine whether expression of wild-type *dNSF* protein can rescue the *comt* phenotype. A P-element transposon bearing a full-length *dNSF* cDNA, under the control of a heat-shock promoter, was used to confer heat-shock-inducible expression of *dNSF* protein. Rescue was assessed by examining the temperature-sensitive paralysis of *comt* flies, which normally occurs within 1–2 minutes of exposure to 38 °C. After heat shock, followed by a 1-day recovery period, *comt* flies bearing the *dNSF* transgene were highly resistant to exposure to 38 °C, while their *comt* siblings lacking the transgene remained sensitive (Fig. 2). Taken

together with the mapping and sequence data, these results show that the *comt* gene is *dNSF*.

Our finding provides new information on the physiological role of NSF. Electrophysiological analysis of neuromuscular transmission in the flight muscles of *comt* mutants<sup>6</sup> showed that nonpermissive temperatures cause a graded decrease in the postsynaptic potential, consistent with a defect in synaptic transmission. Thus, these findings represent the first functional evidence for NSF involvement in synaptic transmission. Further electrophysiological analysis of the *comt* mutant should clarify the role of NSF in this process.

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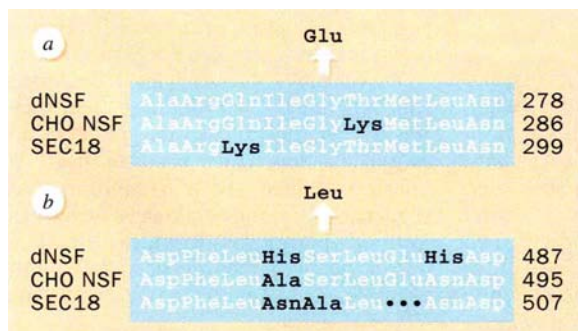
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## Origins of photosynthesis

**SIR** — Nisbet *et al.*<sup>1</sup> in Scientific Correspondence have suggested that photosynthesis originated at hydrothermal vents, where organisms could exploit thermal radiation from the hot water. It is a mystery how primitive organisms could exploit sunlight before the appearance of ozone which provides protection from ultraviolet radiation. Here we attempt to expose some quantitative aspects not mentioned by Nisbet *et al.*

In their computations, Nisbet *et al.* consider water to be a black body. In reality, water itself should radiate more strongly in the absorption bands than at other wavelengths, and these bands are also those which would be most strongly attenuated by the cooler water between the radiation source and the organisms. The radiator does not, however, consist of pure water, and a 'black smoker' would be black enough, so the calculations below are also based on black-body radiation.

To investigate whether thermal radiation from vents would be able to drive biochemical and biophysical processes, I have computed the chemical potential difference that can be achieved using such radiation. As in a previous paper<sup>2</sup> in which I derived the optimum wavelength for the long-wavelength absorption band of a photosynthetic system in unattenuated daylight, I follow the approach of Ross and Calvin<sup>3</sup>. Lands-



**FIG. 1** *dNSF* missense mutations detected by sequence analysis of PCR-derived *dNSF* cDNAs from *comt*<sup>st17</sup> (a) and *comt*<sup>st53</sup> (b) are indicated by the arrows. The relevant portion of the *dNSF* amino-acid sequence is shown in alignment with Chinese hamster ovary NSF and *Saccharomyces cerevisiae* SEC18. Amino-acid identities are highlighted.

MAXIMUM AND EFFECTIVE POTENTIAL DIFFERENCES UNDER A VARIETY OF CONDITIONS

| Long-wavelength absorption maximum of pigment (nm) | Ambient temperature (°C) | Distance of source to organism (cm) | $\mu_o$ (eV) | $\mu_{eff}$ (eV) |
|----------------------------------------------------|--------------------------|-------------------------------------|--------------|------------------|
| 1,020                                              | 4                        | 0                                   | 0.65         | 0.57             |
| 850                                                | 4                        | 0                                   | 0.79         | 0.70             |
| 1,020                                              | 4                        | 15                                  | 0.60         | 0.52             |
| 850                                                | 4                        | 15                                  | 0.73         | 0.65             |
| 1,020                                              | 4                        | 50                                  | 0.47         | 0.40             |
| 850                                                | 4                        | 50                                  | 0.61         | 0.53             |
| 1,020                                              | 100                      | 15                                  | 0.38         | 0.30             |
| 850                                                | 100                      | 15                                  | 0.48         | 0.40             |
| 1,020                                              | 50                       | 50                                  | 0.35         | 0.28             |
| 850                                                | 50                       | 50                                  | 0.48         | 0.40             |

berg and Tonge<sup>4</sup> have developed a more general model, but it is more difficult to understand, and gives the same result as that of Ross and Calvin.

According to Ross and Calvin<sup>3</sup>, the partial molar free energy difference between the excited pigment and the pigment in the ground state, that is, the maximum chemical potential difference that can be achieved by a photochemical system, is given by:

$$\mu_o = kT \ln [\phi_{lum} \int I_s(\lambda) \sigma(\lambda) d\lambda / \int I_{BB}(\lambda) \sigma(\lambda) d\lambda]$$

where  $k$  is Boltzmann's constant,  $T$  is the absolute ambient temperature,  $\lambda$  is wavelength,  $I_s(\lambda)$  is photon fluence rate (scalar photon irradiance) from the source (in this case the hot vent),  $I_{BB}(\lambda)$  is photon fluence rate from thermal black-body radiation at ambient temperature,  $\sigma(\lambda)$  is the absorption cross-section of the pigment of the photochemical system that absorbs the radiation, and  $\phi_{lum}$  is the quantum yield of luminescence for the pigment involved, and is assumed here to be 0.3 (the value for chlorophyll *a*). The integrals should be extended over the whole absorption spectrum of the pigment.

For bacteriochlorophyll *b* the wavelength of the absorption maximum is 1.02  $\mu\text{m}$ , and the half-bandwidth about  $2 \times 25$  nm. I approximate the band by a corresponding gaussian function. Following Nisbet *et al.* I shall first assume  $T_s = 650$  K (377 °C), and an ambient temperature of 277 K (4 °C). With the hot radiation source extending over  $2\pi$  steradians, we then obtain a maximum potential difference ( $\mu_o$ ) of 0.64 eV. This does not deviate much from the value (0.65 eV) obtained if one assumes that the pigment absorbs only at the absorption maximum, so the band shape is not critical in this case.

If the system is to drive a flow of electrons, the potential difference must be lower. Following equation (10) of Ross and Calvin<sup>3</sup>, we obtain a working potential difference for maximum power storage,  $\mu_{eff}$ , of 0.56 eV. Note that this value has been obtained under very generous assumptions, namely that the source extends over  $2\pi$  steradians and intervening water does not absorb any radiation, while ambient temperature is still as low

as 4 °C, a rather unrealistic scenario. For a pigment peaking at 850 nm the corresponding  $\mu_o$  is 0.79 eV and the working potential difference for maximum power storage 0.70 eV.

If we take into account the absorption by water between the source and the organism, the potentials are further decreased. For these calculations I used the absorption coefficients for water of Curcio and Petty<sup>5</sup>. It is, however, quite unreal-

istic to assume an ambient temperature of 4 °C close to a 377 °C source extending over  $2\pi$  steradians. I have also considered the more realistic cases of 100 °C at a distance of 15 cm and 50 °C at a distance of 50 cm (see table). An 850-nm pigment seems to give an advantage over a 1,020-nm pigment; however, the possible power storage is the product of absorbed photon flux and potential difference. Because of the higher spectral irradiance of the source at the longer wavelength, the possible power storage with a 1,020-nm pigment is more than 20 times that obtainable with an 850-nm pigment.

In looking for a plausible start for the evolution of photosynthesis exploiting radiation from hydrothermal vents, I believe we should restrict the search to looking for electron transfer reactions that can be driven by a photochemical system limited to a potential difference of about 0.4 eV. As a comparison, the span between the mid-point potentials for the primary electron donors and acceptors of present-day photosynthetic purple bacteria is about twice this value.

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**SIR** — Nisbet *et al.* in Scientific Correspondence<sup>1</sup> propose that photosynthesis evolved from near-infrared phototaxis that enables chemotrophic bacteria to find hydrothermal vents, as "such places can provide optimal energy that supports life. Organisms that lose touch with such vents risk starvation...".

But that is the situation now, when there is free energy to be gained from combining  $\text{H}_2\text{S}$  from the vent with dissolved  $\text{O}_2$ . Before water-oxidizing photosynthesis first appeared around 2 billion ( $2 \times 10^9$ ) years ago, there was no free  $\text{O}_2$ , and nothing to be gained from occupying dangerously hot places on the ocean floor, as what came out was at redox equilibrium with the ocean. So dismissing "photosynthesis first" on these grounds is a classic example of trying to lift oneself up by the bootstraps.

Even if early chemotrophs gathered at thermal vents to keep warm, there is

another problem: anaerobic photosynthetic bacteria have near-infrared-absorbing bacteriochlorophyll today because this window in the spectrum is made available to them by the rather more successful oxygen-evolvers that need higher-energy (visible) light to split water. Before oxygen evolution, it is likely that the greater energy available from visible light was also used by any photosynthetic organism. So we have another problem, a hidden assumption about what is "primitive".

I prefer to think of anaerobic photosynthetic bacteria as deposited monarchs, waiting, in specialized environments below the thermoclines of lakes, until aerobic republicanism has gone out of fashion. But they are alive today: evolution did not come to a halt in purple bacteria, and they are really rather well adapted to their environment. The emission of near-infrared by hydrothermal vents is more likely to have led to thermotaxis evolving from photosynthesis, I should have thought.

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**NISBET ET AL. REPLY** — Both Allen and Björn raise interesting problems. Allen questions the availability of oxidation power in the early Archaean Earth. This is at present a matter of strong debate. The early atmosphere probably suffered major losses of hydrogen to space (significantly from  $\text{H}_2\text{O}$ , leaving oxygen), but it is likely that despite this oxygen source,  $\text{O}_2$  partial pressure in the troposphere was low. It is probable, however, that an atmosphere-ocean system dominated by  $\text{CO}_2$  and  $\text{H}_2\text{O}$  would have provided oxidation contrast at hydrothermal systems, where the atmosphere-ocean interacts with solutions that have reacted with mantle-derived lava. Thus, hydrothermal systems would indeed have provided habitats for early living communities. This seems to be borne out by the molecular record, which implies that the earliest organisms were exclusively hyperthermophiles<sup>6</sup>. In such a setting, bacteriochlorophyll (which appears to be more primitive than chlorophyll) may have evolved for phototaxis.

We thank Björn for his calculation, which is much more detailed than ours. We stress, however, that we are not proposing that early bacteria lived by infrared photosynthesis from vents. Our suggestion is simply that they developed infrared phototaxis to allow them to detect heat sources at deep-water (for example, mid-ocean ridge) hydrothermal

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vents, which would have been of great selective advantage. Once capable of infrared phototaxis, they were pre-adapted for photosynthesis, and bacteria that accidentally drifted up into the photic zone could then exploit sunlight.

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## Single odorant molecules?

**SIR** — Can vertebrate olfactory receptor cells detect single odorant molecules? Menini *et al.*<sup>1</sup> suggest that under some circumstances this may be so. But we do not think that their paper demonstrates this conclusion.

If, as Menini *et al.* appear to imply, the smallest isolated current bumps in their Fig. 1b (0.5  $\mu$ M cineole trace) were evoked by single molecules, then doubling the odorant concentration should have doubled the number of these events. However, looking at the response evoked by 1  $\mu$ M cineole, it is clear that the number of events was much more than doubled. This discrepancy cannot be explained by nonlinear summation as Menini *et al.* suggested, because nearly all simultaneous events would have occurred in different cilia, and therefore would have summed linearly, given the low rate of 'quantal-like' events in the 0.5  $\mu$ M trace ( $<1$  s<sup>-1</sup>).

A second problem with interpreting the current bumps as single-molecule responses is the extraordinarily high odorant concentration required to evoke "on average very few" such events. During each of the 0.5-s, 10- $\mu$ M cineole stimuli in Fig. 3 of ref. 1, at least 10<sup>8</sup> odorant molecules had access to the cilia by diffusion (calculated by approximating each cilium by a highly elongated ellipsoid of revolution<sup>2</sup>, and assuming a diffusion coefficient of  $5 \times 10^{-6}$  cm<sup>2</sup> s<sup>-1</sup> and 10 cilia, each 30  $\mu$ m long). Yet, according to the interpretation of Menini *et al.*, these stimuli evoked "on average very few" events (two events based on the frequency of "failures"<sup>3</sup>). Thus, interpreting the current bumps as single-molecular responses implies a remarkably low probability of odorant-receptor interaction, contradicting evidence that a single molecule is sufficient to activate transduction<sup>4</sup>.

Finally, the conclusion of Menini *et al.*

depends on the assumption that the current bumps result from quantal activation of transduction, not other sources of noise in the transduction mechanism. We have reported similar current fluctuations in rat olfactory receptor cells, but showed that they reflect noise intrinsic to the transduction mechanism, probably caused by spontaneous fluctuations in the basal cyclic AMP concentration<sup>5,6</sup>. One might assume that intrinsic transduction noise cannot contribute to the measurements of Menini *et al.* because the baseline current exhibits little noise in the absence of odorants. However, this is not true, because the threshold for current generation by cAMP will attenuate basal transduction noise<sup>7</sup>. This threshold can also explain the discrete (quantal-like) appearance of the smallest odorant responses, because fluctuations in cAMP concentration that only rarely exceed the threshold will generate discrete quantal-like current bumps. Such a threshold effect could very well explain the quantal-like appearance of the odorant responses in ref. 1, because the transduction current exhibits roughly a fourth-power dependence on odorant concentration<sup>8</sup>, presumably due to the nonlinearity of current generation<sup>7</sup>. This nonlinearity also undermines their estimating quantal-event amplitude from the histogram in Fig. 3 of ref. 1, because this type of analysis assumes a linear relationship between current and the number of quantal events<sup>9</sup>.

In summary, we believe that the quantal-like current bumps reported in ref. 1 are explained more readily by intrinsic transduction noise than by quantal activation of transduction. In our view, a convincing demonstration of quantal responses must satisfy the same criteria as those that established quantal detection in photoreceptors: linear summation of independent events; Poisson variation in response amplitudes; and agreement between the stimulus magnitude and the number of quantal events<sup>3</sup>. Future studies should also consider the effects of nonlinear current generation and intrinsic transduction noise.

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## Eemian climate and pollen

**SIR** — Field *et al.*<sup>1</sup> have presented quantitative palaeoenvironmental reconstructions for the last interglacial period (the Eemian) in western Europe based on pollen data from two sites (Bispingen in northwestern Germany and Grande Pile in eastern France). In their reconstructions the authors use climate response surfaces and measure the degree of analogy between fossil and modern pollen spectra in order to arrive at their nearest modern analogues. Climate reconstructions were attempted so as to check the pollen evidence for possible Eemian climate oscillations similar to those reported from the GRIP ice core<sup>2</sup>.

The palaeoclimate reconstruction for Bispingen, situated in the German lowlands 50 km south of Hamburg, gave a surprising result. Whereas Eemian summer temperatures were similar to those of the present warm period, winter temperatures during the latter half of the Eemian were very low, with mean temperature of the coldest month (MTCO) averaging  $-10$  °C. This corresponds to present-day temperatures in northern Belarus. During this long period of cold winters there was even an interval with extreme conditions where MTCO decreased to  $-20$  °C, corresponding to the present climate in central Siberia and to supposed temperatures in western Europe during pleniglacial conditions. Present-day MTCO for the Bispingen locality is  $-0.3$  °C.

This palaeoclimate reconstruction is very unlikely in the light of uncontested evidence indicating that during this period of supposed severe winters in the lowlands of western Europe, the North Atlantic Ocean and the Norwegian Sea were open waters at least as warm as those of today<sup>3</sup>. The reconstruction is directly contradicted by the occurrence of pollen of the indicator plants ivy (*Hedera helix*) and holly (*Ilex aquifolium*), which require MTCO well above  $-5$  °C (ref. 4), both in the original Bispingen pollen diagram<sup>5</sup> and in a large number of Eemian pollen diagrams from nearby localities in the lowlands of northwestern Europe<sup>6</sup>.

The discrepancies may be related to difficulties inherent in the technique used for palaeoclimate reconstruction. One main difficulty is to find modern analogues for fossil pollen assemblages. In many cases strict parallels do not exist. In the reconstruction of the Eemian climate in western Europe, this problem is especially acute because one of today's dominant tree species, beech (*Fagus sylvatica*), was not present in the area in previous interglacials. The reason for this absence is not fully known, but does not seem to be climatic<sup>7</sup>. Beech was also absent from



western Europe during most of the present warm period, the Holocene, and only arrived there long after conditions favourable to it were established, perhaps promoted by the introduction of agriculture and associated land-use changes. Nonclimatic shifts in the vegetation constitute a definite source of error in palaeoclimate reconstructions based on modern vegetational analogues. Control by indicator plants is therefore important. Field *et al.* also attempted a climate reconstruction for the Grande Pile location, about 640 km SSW of Bispingen and 330 m above sea level, which today has a MTCO of +0.6 °C. In this reconstruction the interval with extreme winter coldness is absent. This is also the case in the palaeoclimate reconstructions based on Eemian pollen diagrams from Grande Pile by Guiot<sup>8</sup> and Guiot *et al.*<sup>9,10</sup>, in which both MTCO and annual mean temperature during the whole Eemian interglacial are similar to present-day conditions. We therefore suggest that the severe winter spell during the Eemian at Bispingen proposed by Field *et al.* is an artefact caused by limitations in their climate reconstruction method.

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HUNTLEY AND FIELD REPLY — Aaby and Tauber assert that the North Atlantic Ocean and Norwegian Sea were “at least as warm as today” during the period of “supposed severe winter”. Recent high-resolution records of sea-surface temperature<sup>11</sup>, however, show marked cooling in both areas no more than 5,000 yr after the stage 6/5e termination. In the Norwegian Sea, temperatures were similar to those of modern times during only the initial short warm period of 5e, subsequently falling to values comparable to those during glacial stages<sup>11</sup>. In the North Atlantic, early warm period temperatures around 1.7 °C higher than present fell by around 2 °C at the same time<sup>11</sup>. Such a sea-surface temperature scenario is consistent with stronger mid-Eemian cooling in northern and northeastern compared with western and southern Europe and generally concurs with the mechanism we hypothesized<sup>1</sup>. Our hypothesis is also supported by evidence of sea-surface temperature instability in the North Sea during the Eemian; two marked cooling episodes are reported north of Denmark<sup>12</sup>.

Aaby and Tauber suggest that our reconstruction “is directly contradicted” by occurrences of ivy (*Hedera helix* L.) and holly (*Ilex aquifolium* L.) pollen at Bispingen and in other Eemian records, as these taxa are today found only where mean temperature of the coldest month (MTCO) is  $\geq -5$  °C. Careful examination of our reconstruction shows MTCO is

$-5$  °C for approximately 1,500 yr after its fall at a relative age of 7,600 yr, with only one short colder episode during this interval. This is consistent with the palynological evidence of *H. helix* and *I. aquifolium*. Pollen of both occurs consistently in samples during and before this 1,500-yr interval, but is represented extremely sparsely thereafter<sup>5</sup>. The isolated occurrence of both in a sample with relative age about 5,000 yr coincides with a peak in reconstructed MTCO, the upper 95% confidence interval of which reaches  $>-5$  °C. Given that these ‘indicator’ taxa were not used in our reconstruction, their pattern of occurrence provides independent support rather than contradicting our reconstruction. As to their wider occurrences in Eemian records, these can be systematically evaluated only by considering their relative spatial and temporal contexts. We make no claim that the palaeoclimate history reconstructed at Bispingen is universal; indeed, we draw attention to contrasts with sites further south and/or west.

Aaby and Tauber suggest that “the discrepancies” they identify may result from difficulties in finding appropriate analogues. Although, as we indicated<sup>1</sup>, this may apply to the more extreme cold oscillations reconstructed, the degree of analogy generally was comparable to Holocene samples. They further suggest that the absence of beech (*Fagus sylvatica* L.) from northern Europe during the Eemian resulted from unknown but nonclimatic factors and that the resulting difference in forest composition underlies the reconstruction of colder winters than today. While we agree with the latter conclusion, the source cited by Aaby and Tauber in support of their assertions about *F. sylvatica*<sup>7</sup> is contradicted by recent work<sup>13</sup> showing *F. sylvatica* occupies a very similar climatic range to that occupied in eastern North America by *Fagus grandifolia* Ehrh. It has never, to our knowledge, been suggested that human interference has influenced the distribution of the latter. Furthermore, Prentice *et al.*<sup>14</sup> demonstrate that *F. grandifolia* maintained its range in equilibrium with climate

throughout the Holocene. We maintain that the reconstruction of colder winter conditions at Bispingen during the latter two-thirds of the Eemian concurs with the palynological record and is the most parsimonious explanation of observed differences in forest composition between the latter parts of the Holocene and Eemian in this region. The principal features of the reconstruction are consistent with the GRIP ice core data<sup>15</sup>, and with data from ocean and shelf cores<sup>11,12</sup>. The Bispingen region is likely to experience climate changes associated with such changes in circulation and/or surface temperature in the Norwegian and North seas, although there is evidence of Eemian climate fluctuations elsewhere in Europe<sup>16</sup>. Further studies are needed to establish the extent, timing and duration of such fluctuations in different parts of the continent.

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## Lie detection

SIR — The results obtained by R. Wiseman on lie detection, published in *Nature* 373, 391; 1995, but which I read about in the April issue of *Monthly Nature*, must be regarded as dubious, because they were obtained in uncontrolled conditions via polls in the mass media. It is well known that the audience of every newspaper, radio or television station is different. And differences in frequency of lie detection may be the result, not of differences of cues used by observers (visual, verbal, vocal), but of differences in the proportions of skills in the different groups of readers (intelligence, perspicacity and so on).

Another possible source of large bias is the self-selection of people who responded to the request for participation. Numerous television viewers could not detect the lies, fewer *Daily Telegraph* readers could, but many radio listeners were able to detect the lies. This outcome is probably the result of selection, as different proportions of people in the three groups may have failed to respond out of lack of enthusiasm, being too busy and so on. The results obtained by Wiseman can be explained on this basis.

These are only two of the many possible sources of bias in an uncontrolled study of this type.

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# Fantastic archaeology

L. Sprague de Camp

**Fingerprints of the Gods: A Quest for the Beginning and the End.** By Graham Hancock. *Heinemann/Crown*: 1995. Pp. 578. £16.99, \$25.

As most good scientific fringers know, our industrial civilization is not the first of its kind on this planet. Long ago, they say, there thrived another technically advanced culture, enjoying many of the advances that our mechanized world now boasts: electric power, fast transportation, aircraft and so on. But some cosmic catastrophe wiped out this culture, leaving Earth's survivors clinging precariously to Stone Age life. After the passage of thousands of years, human beings slowly climbed out of the Stone Age, especially in Iraq and Egypt, and began the process of developing civilization as we know it all over again.

Some locate this prehistoric civilization in Plato's Atlantis, arousing snorts of derision from geologists and oceanographers, who cite the utter lack of any large submerged land mass in the part of the Atlantic where Plato placed his sunken continent. Others say that the concept and name of 'Atlantis' are not unsuitable if it is understood that the land submerged was actually in Palestine — or Tunisia, or Sri Lanka, or Spitsbergen, or. . .

Graham Hancock goes one better than previous theorizers. He locates his advanced culture in Antarctica, citing the now widely accepted theory that the continents slide around on the Earth's surface. Thus evidence indicates that Antarctica was once a long way from the South Pole. In fact, avers Hancock, it was far enough north to give it a temperate climate. At one time, recent discoveries confirm, it even supported a population of dinosaurs.

This advanced civilization, says Hancock, flourished from about 13,000 BC to 10,000 BC. Then this continent did one of its side-slips around the globe — but not with the glacial slowness that geologists attribute to such movements. This event happened relatively quickly — in hours, days or months, say, instead of over millions of years. It wiped out the civilization of the scientifically advanced Leonians. (I call them that because they are alleged to have flourished in the Age of Leo, just as we flourish at the beginning of the Age of Aquarius. The names refer to the zodiacal signs occupied by the Sun at the vernal equinox.)

This sounds all very exciting: but how do we know that it is true, and not all a mere fictional construct, like Burroughs's Barsoom or Howard's Hyborian Age?

In fact, we do not know, because our knowledge of such long-past ages is fragmentary and contradictory. The best we

can do is to gather together all the evidence we can, painstakingly study it and conclude what probably happened. The more evidence we amass, the smaller becomes the likelihood that 'what happened' differs radically from what the 'orthodox' historians and archaeologists have inferred; but that probability never shrinks to zero.

The Austrian geologist Alfred Lothar Wegener, for example, early this century studied the coast lines of the Americas to the west and of Europe and Africa to the

land ice cap in 1930. This huge discrepancy between Wegener's estimates of time and those revealed by later discoveries long led most geologists to dismiss the idea of continental drift as a mere interesting speculation. Hancock seems to have fallen into the same trap as Wegener.

In pursuit of his revolutionary theory of human history, Hancock has travelled to Peru (to see the Nazca lines and Tiahuanaco) and to Mexico and Egypt. He has calculated that the arrangement of the pyramids of Giza in Egypt and those at Teotihuacan in Mexico were not simply the result of architects' scramble for suitable sites for tombs for their god kings. Their searches were spurred on by the knowledge that, if they did not please the incumbent god-king, he might command: "Off with their heads!"

Moreover, Hancock surmises that the spacing of these ancient monuments sym-

IMAGE  
UNAMBIGUOUS  
FOR A COPY TARGET  
FOR REASON  
REASONS

**Pi in the sky** — Hancock assures his readers that the builders of the pyramids of Giza incorporated into their monuments the value of  $\pi$ , but with enough figures to juggle, anyone can extract cosmic results from unlikely sources.

east. He concluded that all these continents had once been joined together in one great super-continent, for which he proposed the name "Pangaea".

Alas for Wegener. He was also beguiled by a report, which turned out to be grossly mistaken, that claimed that Greenland was moving westward at 60 feet a year. Therefore, argued Wegener, Pangaea began to break up into its component continents only in relatively recent times, shortly before the start of recorded history.

Later, more accurate measurements revealed that the speed of continental drift was much slower than Wegener thought. It was, at most, a few inches or centimetres a year. Although Wegener's theory was basically correct, his Pangaea had begun to disintegrate millions of years earlier than he had thought — in the Mesozoic Era or Age of Reptiles. Later discoveries, such as the periodic reversals of the Earth's magnetism, finally brought the concept of continental drift into general acceptance, nearly half a century after Wegener himself perished on the Green-

bolizes the positions of the three bright stars in Orion's Belt. He is sure that their locations must have been worked out by the mathematically sophisticated Leonians, who flourished in temperate pre-Antarctica. A glance at my current map of Texas shows that the cities of San Antonio, Austin and Dallas enjoy a similar relationship. But I feel sure the Leonians had nothing to do with their placement.

Further, Hancock attributes the Great Pyramid of Giza to his prehistoric Leonians instead of to the generally accepted builder, King Khufu (in Greek, Cheops) of the Fourth Dynasty. Hancock does mention the quarry marks, naming Khufu as the builder, allegedly found by Colonel Howard Vyse in 1837. But Hancock conveniently disposes of this evidence by implying that Vyse was a liar who forged the marks.

Now, for all I know, Vyse may have been as big a liar as Baron von Münchhausen. (I would certainly not trust any discoveries alleged by the late Charles Dawson, the main (although perhaps not

the only) perpetrator of the Piltown hoax.) But what evidence assigns Vyse to this class? Hancock presents none, although such a serious charge calls for evidence.

Hancock also has fun with numbers. He assigns an age to Tiahuanaco of 17,000 years (as computed by a Professor Poznansky) on the basis of the wanderings of the Earth's polar axis and combinations of the numbers of days and years in the stages of the life of the Egyptian god Osiris. He also assures his readers that the builders of the pyramids of Giza and of Teotihuacan incorporated in their circular monuments the value of  $\pi$ , leaving it for us moderns to work out when we attained a state of civilization comparable to theirs.

It has often been shown that, with enough figures to juggle, anyone can extract cosmic results from unlikely sources. Thus Ludwig Borchardt, as an anti-pyramidological joke, derived the base  $e$  of natural logarithms from the slope of Sahura's pyramid. F. A. P. Barnard, by juggling the dimensions of the Temple of Artemis at Ephesus, worked out, he said, the Moon's diameter, the length of the lunar month and the date of the building.

Hancock bolsters his gossamer case by accepting speculative ideas as proven facts as solidly based as the roundness of the Earth. He assures us that Mercator based one of his maps "on the ancient sources which we know he had at his disposal", that Tiahuanaco "was built to serve as a port", that the planners of Giza "knew the earth to be a sphere", and so on. But as Sportin' Life sings: "It ain't necessarily so".

Altogether, this book can at best be recommended to writers of fantasy and science fiction. It is full of ideas usable only as bases for such fiction. But anyone desirous of learning what really happened on Earth thousands of years ago are better advised first to learn what the scientific experts have concluded. True, there is always a chance, although a vanishingly small one, that a world-shaking proposal by some cultist or scientific fringer might turn out to be right after all. Remember poor Wegener! □

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## Making fiction of the truth

John Adams

### But is it True? A Citizen's Guide to Environmental Health and Safety Issues.

By Aaron Wildavsky. Harvard University Press: 1995. Pp. 574. \$35, £21. 95.

In this book, published after his death, Aaron Wildavsky seeks to demonstrate "the feasibility of citizenship in science" — that is, to show nonscientists how they can participate in debates about environmental issues over which scientists disagree. The central question — is it true that our safety, health and environment are being threatened by modern technology? — is of great interest to prime ministers and presidents as well as other less exalted citizens. It is also of interest to scientists reading *Nature*, because debates about such issues go beyond the specialist knowledge of most scientists, leaving them, like the intelligent lay person, struggling to make sense of evidence and arguments that lie beyond their competence.

In earlier books, most notably *Risk and Culture* with Mary Douglas and *Cultural Theory* with Michael Thompson and Richard Ellis, Wildavsky developed a typology of bias; according to this typology and the theory erected on it, people tend to view the world through different cultural spectacles. The typology identified four distinctive perspectives. "Individualists", according to the typology, are optimists;

they believe nature to be benign, bountiful and robust; nature is to be 'commanded' for the benefit of mankind. "Egalitarians" are anxious; the environment is beautiful, but fragile; nature is to be 'obeyed' and interfered with as little as possible. "Hierarchists" trust nature, within limits; they employ scientists to work out where these limits are, and economists to devise strategies for living optimally within them; nature is to be 'managed'. "Fatalists" go with the flow; they have no control over an unpredictable nature; *che sarà sarà*.

Wildavsky's cultural theory describes a world of "plural rationalities" in which 'truth' is elusive; one person's truth becomes another's bias. Wherever and whenever scientific evidence is inconclusive, people are liberated to argue from their prejudices and preconceptions. It is a fundamental tenet of cultural theory that, pending omniscience, we cannot do otherwise. Bias is inevitable; when we suspect danger — but cannot know for sure — we resort to speculation. The 'facts' that inform our speculations pass through cultural filters and are ordered by our predispositions to see the world in particular ways.

*But is it True?* is a guided *tour de force* of most of the American and global environmental *causes célèbres* since "The Great Cranberry Scare" of 1959: dieldrin, saccharin, PCBs and DDT, dioxin, Agent Orange,

Times Beach, Love Canal, Superfund and hazardous waste sites, asbestos, alar, arsenic in drinking water, nitrites, acid rain, ozone depletion and global warming. Wildavsky's method for pursuing the truth about this diverse set of issues involved assigning each issue to one of his students, most of whom did not have science backgrounds. They were told to make what sense they could of the scientific literature, look at what was being said about the subject in the press and on television and find out what governments were doing about it. They discussed their findings with Wildavsky and met in seminars to criticize each other's papers. The papers went through numerous revisions before their arguments were subjected to criticisms by high-powered scientific advisers. Wildavsky insists that this method would work as well for members of garden clubs as it did for his students.

In the penultimate chapter, Wildavsky lays out 20 decision rules that should be followed in investigating charges of environmental harm. The first, and perhaps most important, enjoins the use of controls. Failure to control for such aspects as age, gender, smoking habits, income and diet of the population investigated will, he insists, "gravely weaken" any conclusions about harm that we might draw. His other 19 rules of evidence are equally stringent. But in his conclusion he points to a problem; he notes that there are nearly a thousand different chemicals in a cup of coffee, of which fewer than 30 have been tested for cancer on rodents. The requirement that coffee be proven to be safe is, he complains, an impossible one. But if we apply his rules of evidence, it is almost equally difficult to prove that it is dangerous. The key question, in the face of such uncertainty, is where should the burden of proof lie?

Wildavsky is clear that it should lie on those who fear danger; "in a preventive society", he complains, "the people in charge of raising the alarm will rule." His conclusions, after he applies his rules of evidence, are sweepingly dismissive of environmental alarms: "there are no health benefits from regulation of small, intermittent exposures to chemicals"; "we should assume dose-response relationships to have low-end thresholds — points below which exposures are innocuous"; "other than this one group [high-altitude spruce forests representing less than 0.1 per cent of US forests] there has been no damage due to acid rain"; "ozone thinning is still within the range of natural variation, it could still turn out to be temporary"; "a growing number of scientists argue that increased carbon dioxide . . . is likely to enhance rather than detract from life on earth"; overall "the charges are false, mostly false, unproven, or negligible".

But there is a crucial difference between 'false' and 'unproven'. In some of his case studies, such as dieldrin applied to cranber-

ries, Wildavsky establishes, to my satisfaction, that harm is highly unlikely; in others on his list, however, such as ozone thinning and global warming, he establishes what might be termed 'innocence by association' — wherever guilt cannot be proved, innocence must be assumed.

An unproven verdict throws the argument straight back to the issue of burden of proof. In his earlier work with Douglas, Wildavsky argued that "individuals choose what to fear to support their way of life. . . hierarchists fear social deviance, individualists fear regulation, and egalitarians fear technology." In this book Wildavsky provides abundant evidence that he is an individualist. He enthuses about the benefits of technology to which he gives credit for the developed world's unprecedented level of material well-being, and he dismisses most regulation as an impediment to further progress.

In *But is it True?* he seeks to draw a distinction between 'risk consequences', about which the truth can be known, and 'risk perceptions', which are chosen on the basis of our cultural predispositions. But throughout, his conclusions about what has or has not caused harm are clearly influenced by his application of decision rules that, when the evidence is inconclusive, place the onus of proof on those inclined to believe that harm has been done. For example, when in doubt about the consequences of low-dose toxins, he argues that we ought to assume no effect, whereas egalitarians would argue that we ought to assume the opposite, and hierarchists would commission more research. Our perceptions of risk are clearly derived from our perceptions about what has caused harm in the past.

If the advice proffered in this book were to be universally adopted it would no doubt make for a much better informed debate about environmental issues but, for reasons set out in Wildavsky's earlier work on cultural theory, it would settle few arguments. Long-running debates about the environment are long-running because they are scientifically unresolved. Where evidence about risk consequences is inconclusive, risk perceptions will continue to be rooted in the unscientific soil of faith and prejudice.

Wildavsky describes this book as "self-exemplifying". It exemplifies the wit and vigour that have characterized his previous work. It exemplifies his passion for truth — even in areas where he has shown that it cannot exist. *But is it True?* is a valiant, if ultimately unsuccessful, attempt by Wildavsky to fight free of the bias that his earlier work has convincingly demonstrated is inescapable. It is a splendid advertisement for his unique brand of individualism. □

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## Free thinking

A. J. Meadows

**Out in the Cold: Academic Boycotts and the Isolation of South Africa.** By Lorraine J. Haricombe and F. W. Lancaster. *Information Resources Press, 1110 North Glebe Road, Suite 550, Arlington, Virginia 22201, USA: 1995. Pp. 158. \$29.50.*

A BOYCOTT was originally aimed at individuals or groups who refused to behave in an acceptable way. (The word derives from the name of a nineteenth-century Irish land agent who refused to reduce rents.) At the national level, it has been more usual to speak of applying sanctions to other countries. But sanctions particularly imply economic (or sometimes military) action. So when sanctions are applied to a country such as South Africa, they are often accompanied by a range of other restrictions, which can represent boycotts of specific activities. In this context, an academic boycott is basically a restriction of communication relating to teaching and research. This may be imposed directly by a national government, as happened when the United States banned mail, travel and the supply of publications to Cuba. Although not peculiar to the academic world, the ban clearly implied an academic boycott of Cuba by US scholars. Alternatively, the academic staff may involve themselves directly in a boycott, say by refusing to cooperate in any way with academic staff in the other country.

One of the best-publicized events in the academic boycott of South Africa was of this last type. The organizers of the World Archaeological Congress held at Southampton, England, in 1986 decided to ban all South African participants. The decision proved to be highly controversial: some members of the organizing body resigned and many potential participants decided not to attend. The controversy reflected a basic dilemma that affects academic boycotts. The concept of academic freedom, however battered it has become in recent years, is still widely accepted in universities. It involves the fundamental belief that free inquiry is essential, but only possible without restrictions on individual liberty. From this viewpoint, limitations on academic communication are both a crime and a mistake. The opposing argument is that scholars cannot separate themselves from the countries in which they work: they must interact for better or worse with the wider community. To confuse matters, both viewpoints can be supported from the South African example. Thus the South African Publications Act of 1974 introduced political censorship as a general measure, but it clearly affected the research of a number of South African scholars. But then, university staff did

protest against this act, not least on the grounds of academic freedom.

Although this book contains a good introductory discussion of topics such as these, its main purpose lies elsewhere. It is primarily concerned with examining the impact of the academic boycott (now effectively ended) on scholarship in South Africa. Several hundred questionnaire responses were obtained from academic staff, spread across the various faculties, along with data from a series of interviews. Responses were also obtained from university librarians. The questions to staff concentrated on how the boycott had, or had not, affected them and their sources of information. Overall, more than half the respondents mentioned some effect of the boycott. The most frequent problem mentioned was the refusal by foreign scholars to visit South Africa; a smaller proportion of the respondents experienced difficulties in accessing particular information sources (such as computer programs).

At first sight, these responses seem to suggest that the academic boycott had an appreciable influence. But only about one in ten of the respondents believed that the boycott had had a significant effect on their work. It also seems that the South African universities most active in opposing apartheid were the ones most affected by the boycott. As one respondent remarked: "the academic boycott. . . affected those universities that were fighting apartheid, and suited the universities that were not. . . so the whole academic boycott backfired badly".

Similarly, the South African librarians reported few major difficulties in acquiring material: they could nearly always find alternative sources. In activities such as document supply, which are dominated by a few suppliers, both the United Kingdom and the United States continued to provide a service. (One UK despatch clerk waged a personal campaign by scrawling anti-apartheid slogans on the parcels.) The greatest problems were actually caused by the economic sanctions against South Africa, which led to a fall in the value of the rand, and so to an increasing inability to afford all the material required.

Some factors, as the authors point out, need to be allowed for in looking at the overall analysis. For example, the data may be distorted because, in order to avoid possible embarrassment, some staff in South Africa deliberately did not invite visitors from abroad or travel much themselves. Again, some results were satisfactory — black South African universities, for instance, were less affected than the white. Yet the total impact of the boycott still seems small. This interesting study consequently raises an immediate question: are such boycotts more concerned with reflecting opposition to a regime than with producing specific results? Certainly, the authors' conclusion represents a fair



deduction from their data: "The fact that most participants in our study considered the academic boycott as an irritant and inconvenience, rather than a significant obstacle to scholarly research, does indeed suggest that it was more a symbolic gesture than an effective agent of change." □

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## Distant attraction

Tony Hey

**The Force of Symmetry.** By Vincent Icke. Cambridge University Press: 1995. Pp. 338. £35, \$54.95 (hbk); £13.95, \$24.95 (pbk).

IN a letter to a contemporary about gravity, Isaac Newton wrote that the idea that "one body can act upon another at a distance, through a vacuum, without the mediation of anything else, by and through which action and force may be conveyed from one to the other, is to me so great an absurdity, that I believe no man who has in philosophical matters a competent faculty of thinking can ever fall into it". As is evident from this extract, Newton was clearly deeply uneasy about the mechanism for transmission of the gravitational force between two distant bodies, but the experimental success of his action-at-a-distance force law was undeniable. James Clerk Maxwell had to grapple with the same problems as he struggled to formulate the force laws of electromagnetism. In Maxwell's equations, action-at-a-distance is replaced by an electromagnetic field pervading all space. One of the greatest triumphs of classical physics was Maxwell's prediction of the existence of electromagnetic waves — oscillating electric and magnetic fields travelling with the speed of light. Indeed, light itself was shown to be a form of electromagnetic wave. The world of classical physics now seemed clear: all could be decomposed into charged matter and fields, with the electron as the paradigm of a point-like charged particle.

In this book, the Dutch physicist Vincent Icke takes up the evolution of these concepts in the twentieth century. Less than 50 years after Maxwell first formulated his fundamental equations came disturbing evidence that supposedly classical electromagnetic waves could somehow display particle-like properties. At the same time, our intuitive understanding of the nature of matter also began to fail: supposedly particle-like things such as electrons were shown to display wave-like behaviour. On top of these blows to the self-confidence of classical physics came Einstein and his theories of relativity which forced a radical

change of our ideas about space and time.

This book is about the interplay between quantum mechanics and relativity and about the guiding role played by symmetries in formulating our present view of matter, forces and fields. It is a great challenge that Icke undertakes and one that encompasses explanations not only of the fundamentals of quantum mechanics and relativity but also of the theory of the strong and weak nuclear forces at the frontier of our current understanding. The subject is undeniably fascinating and Icke's emphasis on symmetry and forces is a novel approach in a popular physics book. The only question is whether Icke really succeeds in his ambitious task of covering most of the fundamentals of modern physics at a level suitable for non-physicists.

Let me begin by saying what I like about the book. Icke is clearly an enthusiast whose love of physics is evident throughout. This enthusiasm together with his direct, non-academic style of writing will clearly attract many casual readers with an interest in modern physics. Chapter titles ranging from "Maybe I'm Heisenberg" (for an introduction to the quantum uncertainty principle) to "The physics of a tablecloth" (for an introduction to global and local symmetries) and section headings such as "The dizzy world of spin" and "Strawberry fields forever" give an accurate impression of the style. The early chapters also contain some nice exercises for the reader with a "Feynman diagram scanner" and an experiment with interference fringes. But the more I read, the more unhappy I became with both Icke's approach and style of writing. Part of this unhappiness is no doubt due to my determinedly academic upbringing, but some of my worries seem more than just prejudice. I do not find it helpful to find statements such as "Physics is not difficult; it's just weird", nor do I find it reassuring to be told that physics is "not particularly hard to explain" when there is ample evidence to the contrary. I am also unhappy to find statements such as "Because it is required that  $c + c = c - c = c$ , speed cannot be a simple algebraic number for which the normal rules of addition and subtraction hold" — I know what Icke is trying to explain but believe it unhelpful to write down 'equations' that are patent nonsense.

It was not, however, until his section on "Superposition and quantum sorcery" that I really parted company with his approach. In attempting to explain the difficult and subtle concept of quantum superposition, Icke introduces what he calls a "quantum chimaera, which may be considered simultaneously as one thing and as another". His example of superposition is that of a chimaera made from a pure lion state and a pure goat state. Measurement is described in terms of the chimaera either biting a cabbage or a zebra steak. It is difficult for me to gauge the reactions of a reader not

familiar with quantum mechanics on encountering this analogy, which Icke refers to repeatedly. Suffice it to say that I believe that his lion-goat chimaera is both confusing and unhelpful.

The level of mathematical sophistication required of the reader seems both variable and demanding. The concepts of vectors and vector addition are introduced in a single short paragraph. Phases and amplitudes are reassuringly introduced in the context of wave motion, but Icke then leads the reader on rapidly to complex numbers and Argand diagrams. Despite a footnote reassuring readers that they need not worry in detail about how to 'square' a complex number, a footnote two chapters on gives explicit instructions on how to multiply two complex numbers. It is for pedagogic reasons such as these that I wonder about the audience for the book. Stephen Hawking recounts that he was told that each equation he included in *A Brief History of Time* would halve the sales. In the end, Hawking compromised by including just one equation: Einstein's famous mass-energy relation. Icke includes many equations as well as academic-style footnotes, all of which I suspect will deter all but the most determined seeker after truth.

For the more serious physicist there are several worrying short-cuts taken by Icke in his presentation of quantum physics. I like his tablecloth analogy to explain local and global symmetries, but I am still trying to understand his footnote explaining the use of half-angles for spin-half doublets. His explanation of how to arrive at SU(N) symmetries makes it seem that theoretical physicists were breathtakingly stupid in not realizing this for 30 years or more. His dismissal of the problem of "collapse of the wave packet" as "a frightful misnomer" ignores problems that great quantum physicists such as John Bell spent a lifetime taking seriously. There are many other examples of such misleading oversimplifications, but one last one will make the point. His discussion of Heisenberg's uncertainty principle suggests that the momentum-position and energy-time uncertainty relations are on the same footing: Icke then goes on to talk about conjugate variables and operators. Energy and time are not conjugate variables in quantum mechanics. The famous Russian physicist Lev Landau was fond of illustrating that there was no restriction on the accuracy of their simultaneous measurement by saying: "There is obviously no such limitation — I can measure the energy and look at my watch; then I know both energy and time!" The correct interpretation of the energy-time uncertainty relation requires careful discussion: such care is unfortunately often lacking in this book. □

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# Pattern recognition computation using action potential timing for stimulus representation

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**A computational model is described in which the sizes of variables are represented by the explicit times at which action potentials occur, rather than by the more usual 'firing rate' of neurons. The comparison of patterns over sets of analogue variables is done by a network using different delays for different information paths. This mode of computation explains how one scheme of neuroarchitecture can be used for very different sensory modalities and seemingly different computations. The oscillations and anatomy of the mammalian olfactory systems have a simple interpretation in terms of this representation, and relate to processing in the auditory system. Single-electrode recording would not detect such neural computing. Recognition 'units' in this style respond more like radial basis function units than elementary sigmoid units.**

HERE I describe a recognition problem that occurs in many guises and sensory modalities, and that can be efficiently solved when analogue information is encoded (or represented) using action potential timing. I emphasize the importance of neurobiological information representations for their ease in computing useful results, rather than viewing the choice of representation as chiefly a means efficiently to transmit 'information' as defined in communication theory<sup>1-3</sup>.

The choice of information representation is of vital importance in making a computation easy, as is illustrated by the question 'is 3630225 divisible by 7?'. If the number 3630225 is in base 10, answering this takes a bit of work. If the number 3630225 is in base 7, the answer is immediate from inspection of the last digit. The choice of representation for analogue information is equally important for neural computation.

In this paper, analogue information is represented by using the timing of action potentials with respect to an ongoing collective oscillatory pattern of activity. This is a long-hypothesized representation<sup>4</sup> for which there is now appreciable evidence<sup>5</sup> for special cases. Experiments<sup>6</sup> on rat hippocampal 'place cells' indicate that the phase of neural activity with respect to the ongoing local theta-rhythm correlates with the location of the rat within that receptive field. Action potential phase-time coding is also found in electric fish<sup>7</sup>. The particular representation of analogue information used here is created by elementary cellular biophysics in a neuron having a subthreshold oscillatory membrane potential. In contrast, most previous descriptions of neurobiological computation are based on either firing-rate coding or place-cell coding of analogue information.

The scheme computes in this analogue representation by combining information through pathways with different delays. This mode of computation is believed to be responsible for the movement-sensitive visual neurons<sup>8</sup>, and for spatial localization of sounds in the barn owl<sup>9</sup>. The analysis requires a coherence of the oscillation across a localized set of neurons, but such coherence is common<sup>5</sup> at frequencies ranging from 1 to 100 Hz.

Thus the major basis phenomena needed in this new analysis are all known to occur in neurobiology. These known components, working together, can rapidly and efficiently perform computations that are essential to pattern recognition, and that are much more difficult to perform in a rate-coding framework.

I analyse the problem of 'analogue pattern recognition'. This problem, in slightly different guises, occurs in the recognition of

colours, visual patterns, odours and sound quality, and is a very general task which neurobiology solves rapidly. Surprisingly, this problem is not easily solved by most rate-code neural models.

## Calculating analogue match

When a stellar constellation is pictured on the cover of a magazine we ask ourselves whether it is Orion or Ursa Major? Is a faint smell from a recently opened bottle of Bordeaux? These disparate questions are examples of analogue match problems. The basic stimulus is a pattern of analogue values, the ratios and scale of which define the stimulus quality and intensity. For example, the ratios of the lengths of the lines connecting the different pairs of stars determine the shape of the constellation, while the length scale of the lines describes the size of the photographic rendering. In the olfactory example, the currents generated in the individual neurons (or classes of neurons) are the analogue pattern. Sensory neurons in the general olfactory system are not highly specific, and a particular odour must be identified by its pattern of strength of excitation over the ensemble of primary sensory neurons<sup>10</sup>.

The computational problem can be posed as follows. Let a nervous system have implicit knowledge of several stimuli  $a, b, c, \dots$ , each one characterized by a list of numbers  $X_a = \{X_{a,1}, X_{a,2}, \dots, X_{a,i} \dots\}$ . An unknown stimulus is presented which has the properties  $X_u$ . We wish to determine for which (if any) of  $a, b, c$  can it be considered that

$$X_u \sim \lambda X_a \quad \text{or} \quad X_{u,i} \sim \lambda X_{a,i} \quad \text{for all } i \quad (1)$$

and also the value of  $\lambda$ . The form (1) implies scale-invariant recognition of a stimulus quality and knowledge of its intensity or size.

A simple conventional neural model has difficulties in solving this problem. Consider a single 'grandmother cell' neuron which is to recognize a single odour, and distinguish it from other, perhaps unknown, odours. The neuron has a monotonic input-output relationship such as

$$V_{\text{out}} = 1/(1 + e^{-v_{\text{in}}}); \quad v_{\text{in}} = v_{\text{bias}} + \sum W_i X_{u,i} \quad (2)$$

The  $W_i$  are the 'synaptic weights' of the problem;  $V_{\text{out}} > 0.5$  might be taken as recognition of the odour<sup>11</sup>.

This elementary system is not satisfactory because any odourant  $u$  for which the scalar product  $W \cdot X_u$  is positive will, if strong enough, be recognized as the known odour. A system which separates odour quality (which involves only the relative components of  $X$ ) and odour intensity, and judges the match of odour quality independently of the scale factor, is essential. Preprocessing the input  $X$  through a network which converts  $X$  into a vector of fixed length is one way to solve the problem. Conceptually, this requires the computation of the euclidian length of the raw input vector, and then division of all inputs by that factor. This mathematics is not natural to elementary neural circuits.

This structure of network, even with normalization, has two additional undesirable features. First, sensitivity to minor components is lost. If the known odour had intensity ratios 5:5:1 for three variables, the optimal weights to recognize that odour correctly are also in the ratio 5:5:1, so the third component is multiplied by a small weight, and is not a substantial contributor to  $v_{in}$  even if it is of great significance.

An additional problem is involved if we attempt to split the recognition problem into sub-parts. If the raw inputs are split into two groups, and each is processed by separate networks of this type, the outputs of the two 'grandmother units' cannot simply be added to gain confidence in a recognition. For if the two units both seem to recognize the odour, but the scale factors described by the two normalizing processes are very different, the overall pattern is not a good match.

The encoding and computation model described in the next sections uses action potential timing to carry information. It further differs from the above (1) in solving analogue pattern recognition problems simply and naturally; (2) in allowing problems to be broken into smaller sub-parts and the results then combined; and (3) in embedding the information about the pattern to be recognized in a pattern of axonal or cellular time delays. Synaptic strengths determine only the importance of particular variables, not the pattern to be recognized, and therefore require little precision. The processing is intrinsically rapid.

### Time-encoding information

The model involves 'encoding neurons' which produce action potentials, and which are also influenced by an oscillatory drive. (In the vertebrate olfactory system, these units might correspond to the mitral cells of the olfactory bulb.) In the model, a cell will produce an action potential at time  $t$  if the cell potential  $u(t) > u_{\text{thresh}}$  and if no action potential has been generated more recently than the refractory period  $\tau_r$ . It is not necessary to consider the detailed biophysics of action potentials.

The other essential feature of the model is an oscillatory subthreshold variation of the membrane potential for the encoding neurons. Such an oscillatory potential can be generated by intrinsic cellular effects<sup>12,13</sup> or multineuron circuitry<sup>14</sup>. The encoding cells are also presumed to have a time constant which is somewhat shorter than the oscillation period. The cell potential of encoding cell  $j$  (except for the rapid action potential component) will thus be taken to be

$$(u_j(t) - u_{\text{thresh}})/R = I_j(t) - I_0 - A(1 - \cos 2\pi ft) \quad (3)$$

where  $A$  and  $I_0$  are positive constants,  $R$  is the cell resistance,  $I_j(t)$  is the input current to cell  $j$ , and  $f$  is the frequency of subthreshold oscillation. For simplicity,  $I_j(t)$  is assumed to be excitatory, but inhibitory schemes can also be developed. The cosine function is used for illustration; in general, the periodic oscillation will have a more complex form. In the absence of an input current  $I_j(t)$ , the cell exhibits only subthreshold membrane potential oscillations. When the input current exceeds  $I_0$ , action potentials will be generated. The dead time and level of input can together prevent the cell from generating two action poten-

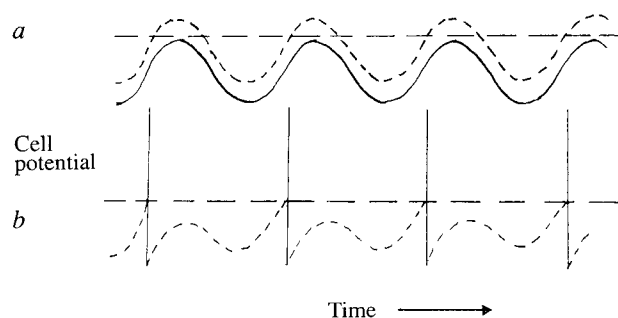


FIG. 1 *a*, When there is no input current, the subthreshold cell potential oscillation (solid line) never exceeds the firing threshold (horizontal broken line). When an input current is added, the cell potential (neglecting currents which flow during an action potential) will cross threshold, as shown by the broken curve. *b*, The cell potential corresponding to the broken curve in *a*, including the effect of the currents which flow during action potentials, which leave the cell in a depolarized state from which it slowly recovers. The cell potential never again exceeds the threshold for spike generation until the next cycle of the periodic oscillation. Thus the cell fires only on the upward threshold crossings of the broken line in *a*.

tials within one period of the oscillation (Fig. 1). The way several cells respond to an analogue input pattern is shown in Fig. 2. The time  $\tau_j$  each neuron  $j$  fires in advance of the maximum of the subthreshold oscillation is determined by its input current, thus encoding the analogue information  $I_j$  as a time advance  $\tau_j$ . The firing frequency for all active neurons is simply  $f$ .

The functional form of the time advance as a function of the input current is needed for further analysis. This is described by a function  $\tau(I)$ , which is determined by the shape of the oscillatory part of the cell potential and any nonlinear transformation which may have been performed on the inputs (such as the logarithmic intensity transformation in the visual system<sup>15</sup>). I assume in the following section that the preprocessing of the fundamental sensory variable  $X$  has been matched to the shape of the top of the oscillatory cycle to yield a net encoding such that signal input channel  $j$  has an advance

$$\tau_j = \sim \ln(X_j/\delta + 1) = \sim \ln(X_j/\delta) \quad \text{for strong odours} \quad (4)$$

where  $\delta$  is a constant scale factor.

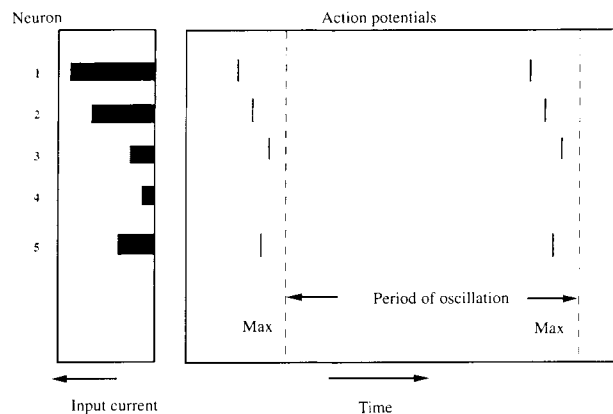


FIG. 2 The action potential of five different neurons driven with different input currents (analogue signal strengths). Neuron 4 produces no action potentials because the input current is insufficient to ever drive that cell above threshold. The other neurons all fire once during each oscillation period, but the time  $\tau_j$  each neuron  $j$  fires in advance of the maximum of the subthreshold oscillation is determined by its input current, and is larger for neurons driven with a greater input current. The firing frequency remains  $f$  over a considerable range of input current if the refractory period is long.

It has so far been presumed that a neuron never fires more than once during a cycle, which will be the case within a limited regime of input current, frequency, postspike depolarization and cell time constant. Outside this regime, the first action potential will occur at the location described, but additional action potentials may also be generated within a period. In this case, the first spike will carry appropriate analogue information on its timing, and analogue information will also be carried in the number of action potentials which occur within a cycle. Thus more than one representation of analogue information can be simultaneously present. Rat hippocampal cells<sup>6</sup> seem to respond in such a dual fashion.

### Decoding and recognition

When a cell has a relatively short integration time constant, its response to action potentials arriving from different afferents is sensitive to the relative times of arrival of the action potentials. A cell can thus serve as a 'coincidence detector' for action potentials impinging on it by different pathways. The auditory place cells found in the barn owl<sup>9</sup> are believed to 'compute' azimuthal position of a sound source by detecting a time coincidence between signals which appear delayed with respect to each other by the different lengths of the paths between the sound source and each ear, and then have compensating axonal delays which result in a coincidence of arrival on a target cell. The decoding or recognition scheme to be described is a generalization of this idea.

More generally, when a pattern consists of a set of features occurring in a given time relationship it can be recognized by a processing system which uses time delays<sup>16</sup> and coincidence detection. These time delays are organized so that the features recognized by feature-detecting neurons, although occurring at different times, produce signals which arrive simultaneously at a recognition neuron which then responds maximally. This motif is found in visual systems that detect the directional movement of edges<sup>8</sup>. This delay organization is believed to be how the moustache bat processes FM sonar echoes<sup>17</sup>. The biophysical mechanism of delay can be synaptic, axonal or cellular. Time-delay decoding is a powerful engineering tool, and has been successfully used in recognizing words in continuous natural speech<sup>18</sup>.

In biosonar an animal generates a scanning sound, and the reflected sound has two kinds of information. The relative times of the received sound features describe the object being detected, whereas the overall time of the return (with respect to the scanning sound) represents a totally different but significant variable, the distance to the reflecting object.

In the examples cited above, the time pattern to be decoded originates with the nature of the time-dependent stimulus. By contrast, the time pattern to be decoded in the present case originates with the encoding scheme itself, and is created even for a constant input stimulus. However, a pattern in time can be decoded by a network of coordinated time delays, regardless of its origin.

Logarithmic encoding (see equation (4)) is particularly useful, because it makes the relative timing of the action potentials encoding a pattern independent of the intensity or scale. If the strength of an odour is increased by a factor of 2, each action potential will be advanced in time by the same amount, and the (time-shifted) pattern will be recognized in a scale-invariant fashion by a time-delay network. The recognition event for the stronger case will simply occur earlier with respect to the maxima of the oscillatory potential. Information about the scale of a recognized pattern is thus encoded in the time at which the recognition event occurs in a 'grandmother cell' or network. A recognition can be readily broken into two parts and recombined as follows. The inputs are divided into two groups which are independently coded and recognized by time-delay networks. If these two independent recognitions correspond to the same intensity, they agree and support each other. If they correspond

to different intensities, then the overall pattern is wrong. However, if they correspond to the same intensity then the two recognition neurons will generate action potentials at the same time; otherwise they will not. Coincidence detection is all that is needed to recombine the parts and to see if the two partial results support each other.

If input current levels are so high that multiple spikes are present in a single cycle, the correct recognition will still be made. However, in addition, spurious recognitions due to these other spikes might sometimes be made later in the cycle, but later recognitions might be discriminated against. Alternatively, synaptic suppression with an appropriate recovery time could chiefly suppress the effect of multiple spikes within a single cycle. Multiple spikes within a single cycle will therefore not debilitate this mode of computation.

### Comparison with mammalian olfaction

The three features necessary to use this representation for recognizing odours are present in the mammalian olfactory system. The olfactory bulb, which is the earliest processing area, shows pronounced global oscillations at about 40 Hz<sup>19</sup>. Thus the mitral cells of the olfactory bulb, which receive inputs directly from sensory cells and send axons directly to the piriform cortex<sup>20</sup>, could encode the input current by the mechanism described. The pyramidal cells in the piriform cortex receive inputs from the axon tract from the olfactory bulb, and from the association fibres within the piriform cortex itself. The anatomy and very slow propagation velocities generate a distribution of time delays as long as 20 ms across the rat piriform cortex<sup>21</sup>. This anatomy is well suited to decoding the kind of analogue coding we have suggested, but makes no sense if the information arriving is primarily rate coded.

The response of principal neurons in the olfactory bulb to the presence of odours is unexpectedly complex<sup>22</sup>. In an elementary model of odour processing, a mitral cell in the olfactory bulb might be expected to respond strongly to one odour and weakly to another, amplifying the discrimination between odours which is begun by the broadly tuned sensory neurons. Although some selectivity is seen, the response of many mitral cells also strongly depends on concentration and on the timing within the breathing and oscillation cycle. This mitral cell response may be an indication of a very different way of encoding analogue information.

Oscillation occurs in other olfactory systems as well. The locust<sup>23</sup> antenna ganglion shows coherent oscillation at about 20 Hz, whereas the procerebral lobe of the slug<sup>24</sup> shows field potential oscillations at about 0.5 Hz. Many reasons for the oscillation in olfaction have been suggested, ranging from an epiphenomenon of the circuitry to explicit uses such as enhancing differences between signals<sup>25</sup>. The scheme I outline here is different from these in that the function of the oscillation, to recode analogue information into the time domain, cannot readily be done by more conventional means.

### Discussion

The model described here is a particular example of a general idea, using action potential timing to encode analogue information, and using time-delay networks to compute in this representation. Such a coding system has great computational power and speed compared with a network using an 'instantaneous rate' coding of variables. A calculation of analogue pattern match can be done in 25 ms with neurons that have firing rates of no more than 40 Hz.

The cell model, the coding and the decoding networks are overly simple and without feedback pathways, so a correspondence between the described model and electrophysiology cannot be completely direct. However, the viewpoint does explain many of the enigmatic features of olfactory systems.

The use of such computation could be widespread. Much of the palaeocortex shares the architecture of the piriform cortex<sup>26</sup>,



and if piriform cortex computes through time-delay representations, so might other parts of the palaeocortex. The frontal cortex in higher mammals, where cells typically have very low firing rates, is another obvious candidate for the use of timing as a means of representing information.

Elaboration on this basic theme could involve more complex subthreshold behaviour, or neurons with more complicated ways of transforming analogue variables to time sequences, and multiple action potentials during a single oscillation cycle. I have described only the simplest of possible encodings. The important general point is the combination of speed and ease of computation with a time-domain representation of analogue variables, and the different point of view such ideas bring to experiments. Understanding how neurobiology functions requires an understanding of both how information is encoded and how that encoded information can be used in a subsequent computation (decoded)<sup>5</sup>.

Single-electrode recording is completely incapable of elucidating the nature of this new representation/computation model. Because weakly driven cells do not respond at all, and strongly driven cells all fire at the same frequency, conventional electrophysiology would simply conclude that the cells are broadly tuned. The existence of such a simple and neurobiologically plausible alternative computational model underlines the potential importance of multielectrode experiments.

Synchronized action potentials play a very different role in the processing described here from that which they have recently been hypothesized to play in the visual system<sup>27,28</sup>. If information is encoded as hypothesized, then neurons that receive little input fire with very little time advance (or none at all), and will appear synchronized. These are generally the neurons with the least useful information about an odour. The neurons representing the strong components of the odour fire earlier in time and will not appear synchronous.

The dynamic range and accuracy of pattern recognition in such a scheme depend on the time resolution available. Azimuthal binaural sound localization by owls has an angular accuracy of 1°, corresponding to a time delay of 20 µs<sup>29</sup>. The 25-ms characteristic time of a 40 Hz oscillation leaves a lot of room for resolution and dynamic range if the neural architecture has been optimized for time-delay processing. If the 5 Hz theta-rhythm is used for encoding, slower synapses would be adequate for decoding. This representation of analogue information does not require synapse strengths of great precision, because the stored analogue patterns against which the incoming information is to be compared are contained in the time delays. The

strength of a synapse reflects the importance given to a particular input variable, not the size of a variable.

When sensory signals themselves have time-dependent structure, the analogue value to time-delay computations can be used without the need for an auxiliary oscillatory pattern. Binaural sound localization in the bat shows some indications of computation of intensity to time delay. Binaural cells in the medial superior olive are optimally tuned both in time delay between the two ears and in an intensity difference, with greater relative sensitivity to intensity difference. Harnischfeger *et al.*<sup>30</sup> note that "for usual latency reasons, and the way sound sources should behave (shadowing) time delay and intensity go hand-in-hand". That is, a network that can work with binaural time delay for azimuthal localization of sound would naturally also function on the basis of intensity differences, because elementary cellular biophysics results directly in time delays resulting from stimulus intensity differences. This system can therefore also be understood to use intensity-to-time-delay encoding and processing, except that in this case an oscillatory potential is not needed. Intensity-to-time encoding in the olfactory system might also be used without oscillations in conjunction with exploratory behaviour such as sniffing or antenna waving, which could serve as the onset for intensity-to-time encoding on a different timescale and at the level of the sensory cells. In this regard it is interesting to note that olfactory sensory cells in the frog have different time responses for different stimuli<sup>31</sup>.

If this kind of computation takes place in neurobiology, the time delays appropriate to a new stimulus must be learned. The simplest scheme is to have a multiplicity of time delays, direct or indirect, available via different synapses. This would be easily done in a structure like the piriform cortex, where the recurrent collaterals allow many possible indirect paths of different time delay between two cells. In such a structure the correct time delays would merely need to be selected by strengthening appropriate synapses using a Hebb-like rule. Alternatively, when synaptic delays are important these delays might be modified when the pre- and postsynaptic cells fired in near synchrony.

Computer scientists have noted that 'radial basis function units', the response of which is maximal for the chosen template pattern, are often better for engineering pattern-recognition networks than are the simple sigmoid response units<sup>32,33</sup> so often used in modelling. The time-delay encoding of analogue patterns results in the units of a time-delay decoding network also having maximal response for a template analogue pattern. Thus neurophysiology can generate this powerful radial basis-function behaviour in a simple feedforward network. □

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# Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis

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**The protease responsible for the cleavage of poly(ADP-ribose) polymerase and necessary for apoptosis has been purified and characterized. This enzyme, named apopain, is composed of two subunits of relative molecular mass ( $M_r$ ) 17K and 12K that are derived from a common proenzyme identified as CPP32. This proenzyme is related to interleukin-1 $\beta$ -converting enzyme (ICE) and CED-3, the product of a gene required for programmed cell death in *Caenorhabditis elegans*. A potent peptide aldehyde inhibitor has been developed and shown to prevent apoptotic events *in vitro*, suggesting that apopain/CPP32 is important for the initiation of apoptotic cell death.**

APOPTOSIS constitutes a systematic means of cell suicide within an organism during normal morphogenesis, tissue remodelling and in response to pathogenic infections or other irreparable cell damage. Inappropriate apoptosis may underlie the aetiology of human diseases such as Alzheimer's, Parkinson's and Huntington's diseases, immune deficiency and autoimmune disorders, ischaemic cardiovascular and neurological injury, alopecia, leukaemias, lymphomas and other cancers, which therefore makes the control of apoptosis an important potential target for therapeutic intervention<sup>1-4</sup>.

Several of the biochemical events that contribute to apoptotic cell death have recently been elucidated. Genetic evidence in nematodes, for example, has identified both positive and negative regulators of apoptosis<sup>5</sup>. The key pro-apoptotic gene, *ced-3*, encodes a putative cysteine protease that is related to mammalian interleukin-1 $\beta$ -converting enzyme (ICE)<sup>6</sup>, the first identified member of a new family of cysteine proteases with the distinguishing feature of a near-absolute specificity for aspartic acid in the S<sub>1</sub> subsite<sup>7,8</sup>. Deletion or mutation of the *ced-3* gene completely prevented the apoptotic death of all cells that were otherwise destined to die, and both CED-3 and ICE induced apoptosis when transfected into a variety of host cells<sup>6,9,10</sup>. Furthermore, the pro-apoptotic effects of CED-3 could be prevented by the nematode cell-death suppressor gene *ced-9*, and to some degree by its mammalian counterpart, the proto-oncogene *bcl-2*. The fate of eukaryotic cells may therefore reside in the balance between the opposing pro-apoptotic effects of an ICE/CED-3-like protease and an upstream regulatory mechanism involving Bcl-2 and/or its homologues.

One of the potential substrates for an ICE/CED-3-like protease during apoptosis is poly(ADP-ribose) polymerase (PARP), an enzyme that appears to be involved in DNA repair, genome surveillance and integrity<sup>11-17</sup>, predominantly in response to environmental stress<sup>18</sup>. PARP is proteolytically cleaved at the

onset of apoptosis by a hitherto-unidentified protease with properties that resemble those of ICE<sup>19,20</sup>. The cleavage site within PARP (DEVD 216-G 217) resembles one of the two sites in proIL-1 $\beta$  (FEAD 27-G 28) that are recognized and cleaved by ICE. Proteolytic cleavage of PARP at this site results in the separation of the two zinc-finger DNA-binding motifs in the amino terminus of PARP from the automodification and poly(ADP-ribosylating) catalytic domains located in the carboxy terminus of the polypeptide. This cleavage precludes the catalytic domain of PARP from being recruited to sites of DNA damage, and presumably disables PARP from coordinating subsequent repair and genome maintenance events. Furthermore, the Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease implicated in the internucleosomal DNA cleavage that is a hallmark of apoptosis is negatively regulated by poly(ADP-ribosylation)<sup>21-23</sup>. Loss of normal PARP function may therefore render this nuclease highly activated in dying cells.

The five known members of the ICE/CED-3 family of cysteine proteases which are of human origin are ICE, ICE<sub>rel</sub>-II, ICE<sub>rel</sub>-III, Nedd-2/ICH-1 and CPP-32 (refs 24-27). Each is capable of initiating an apoptotic response when transfected into host cells; however, it is possible that overexpression of any protease may cause nonspecific induction of cell death. Cytoplasmic expression of other proteases, such as trypsin, chymotrypsin, proteinase K or granzyme B, for example, have also been shown to induce apoptosis<sup>28,29</sup>.

Here we demonstrate that an active form of CPP-32, for which the name apopain is proposed, is the enzyme responsible for the specific proteolytic breakdown of PARP that occurs at the onset of apoptosis. Furthermore, we show that inhibition of CPP-32/apopain-mediated PARP cleavage attenuates apoptosis *in vitro*, demonstrating the importance of this protease in the apoptosis of mammalian cells.

## PARP cleavage activity

An early event that occurs concomitantly with the onset of apoptosis is the proteolytic breakdown of PARP by an unidentified protease with properties resembling those of ICE (prICE)<sup>20</sup>. The resulting cleavage (between Asp 216 and Gly 217) separates the N-terminal DNA-nick sensor of PARP from its C-terminal catalytic domain (Fig. 1a). To measure this proteolytic activity, [<sup>35</sup>S]PARP was generated as a substrate by *in vitro* transcription/translation of a full-length human PARP complementary DNA clone and was then combined with various cell extracts (Fig. 1b). A human osteosarcoma cell line with a propensity for spontaneous apoptotic death contained substantial PARP cleavage activity that was markedly higher in extracts from apoptotic cells versus non-apoptotic cells (lanes 2 and 3, respectively). Osteosarcoma cells are a good model system for studying the events that occur during apoptosis. Upon reaching confluence in culture they undergo the morphological and biochemical changes characteristic of apoptotic death, including cell shrinkage, membrane blebbing, chromatin condensation and fragmentation (not shown), as well as internucleosomal DNA cleavage (Fig. 1c). Coincident with the progressive apoptosis that occurred in postconfluent osteosarcoma cell cultures, the PARP cleavage activity measured in cell extracts was elevated more than tenfold (Fig. 1d). There was no detectable ICE in these extracts, as judged by immunoblotting, reverse-transcriptase polymerase chain reaction (RT-PCR) (not shown), and by the absence of proIL-1 $\beta$  processing, indicating that ICE is not necessary for apoptosis or for PARP cleavage. The lack of a major role for

ICE in apoptosis was confirmed in ICE-deficient mice where apoptosis largely occurred normally<sup>30,31</sup>, although a potential role for ICE in Fas-mediated thymocyte apoptosis has been suggested<sup>32,33</sup>.

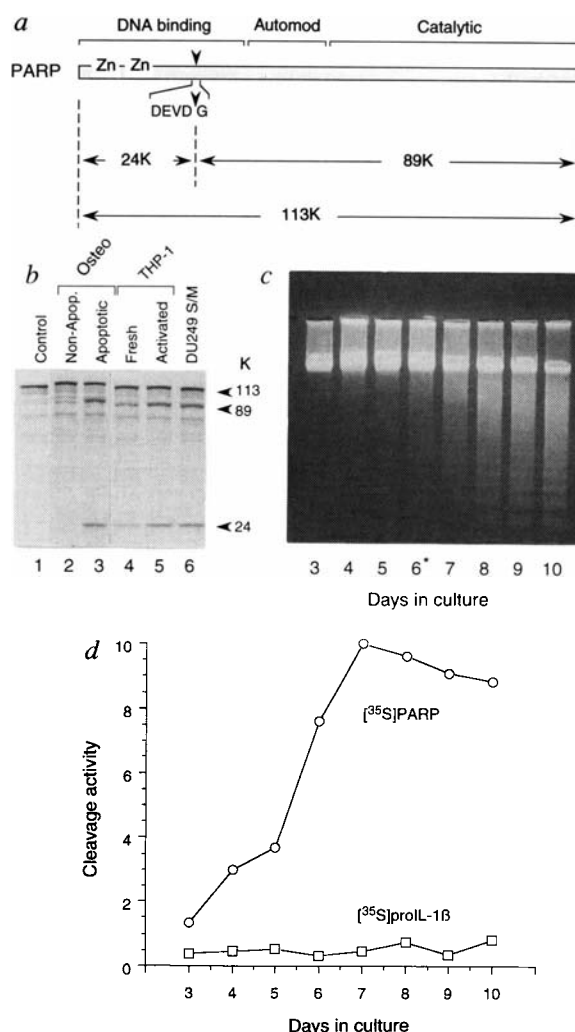
PARP cleavage activity was also measurable in cytoplasmic extracts of THP-1 cells, the human monocytic leukaemia cell line from which ICE was originally purified, particularly after pre-incubation of the extracts at 37 °C (Fig. 1b, lanes 4 and 5). This suggests that the PARP cleavage enzyme requires activation of a latent form, as has been described for ICE in this cell line<sup>34</sup>. PARP cleavage in apoptotic osteosarcoma cell extracts and activated THP-1 cell extracts was comparable to that in apoptotic chicken S/M extracts (lane 6; S/M extracts were prepared from chicken hepatoma cells committed to apoptosis by sequential synchronization in the S phase and mitosis), where this proteolytic activity was originally identified<sup>20</sup>.

## Inhibitors of PARP cleavage

The proteolytic cleavage of PARP in cytosolic extracts from apoptotic osteosarcoma cells was abolished by the cysteine-alkylating reagents *N*-ethylmaleimide and iodoacetamide, but not by E-64 (also a cysteine-protease inhibitor) or by inhibitors of serine-, aspartate- or metallo-proteases (Fig. 2a). This inhibitor profile is consistent with the PARP cleavage enzyme being a member of the emerging ICE-like family of cysteine proteases. To develop more potent and specific PARP cleavage inhibitors, the sequences proximal to the scissile bond were used as a template for inhibitor design. Appropriate peptide aldehydes can be

FIG. 1 PARP cleavage activity in spontaneously apoptotic osteosarcoma cells. **a**, Structure of PARP<sup>42</sup> and fragments resulting from proteolytic cleavage<sup>20</sup>. **b**, *In vitro* cleavage of PARP by cell extracts. [<sup>35</sup>S]PARP was generated by *in vitro* transcription/translation then combined with cytosolic extracts from pre-confluent, non-apoptotic osteosarcoma cells (4.5  $\mu$ g, lane 2), postconfluent, apoptotic osteosarcoma cells (4.5  $\mu$ g, lane 3), freshly isolated THP-1 cells (30  $\mu$ g, lane 4), THP-1 cell extracts activated by preincubation for 60 min at 37 °C (30  $\mu$ g, lane 5), or apoptotic chicken S/M extracts (0.6  $\mu$ g, lane 6). **c**, Internucleosomal DNA cleavage in progressively apoptotic osteosarcoma cells. Osteosarcoma cells were maintained in culture for the indicated number of days and then collected. DNA was extracted and resolved on agarose gels. An asterisk indicates the time point (day 6) where confluence was reached. **d**, Elevation of PARP cleavage activity in progressively apoptotic osteosarcoma cells. Cytosol fractions were isolated from cells described in **c** and then assayed for PARP cleavage activity (circles) and proIL-1 $\beta$  cleavage activity (squares).

**METHODS.** Cytosolic extracts were prepared from cultured human osteosarcoma cells (143.98.2; ATCC CRL 11226) and THP-1 cells (ATCC TIB 202) by homogenizing PBS-washed cell pellets in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride, 10  $\mu$ g ml<sup>-1</sup> pepstatin A, 20  $\mu$ g ml<sup>-1</sup> leupeptin, 10  $\mu$ g ml<sup>-1</sup> aprotinin (at  $1 \times 10^8$  cells ml<sup>-1</sup>) and recovering the post 100,000g supernatant after centrifugation. Chicken S/M extracts were prepared from DU249 hepatoma cells<sup>43</sup> that were committed to apoptosis by S-phase aphidicolin arrest followed by M-phase accumulation with nocodazole as described previously<sup>41</sup>. The full-length cDNA clone for PARP (pcD-12)<sup>44</sup> was excised and ligated into the *Xho*I site of pBluescript-II SK+ (Stratagene) then used to drive the synthesis of [<sup>35</sup>S]methionine-labelled PARP by coupled transcription/translation. [<sup>35</sup>S]PARP cleavage activity was measured essentially as described previously<sup>7</sup> for ICE-dependent cleavage of [<sup>35</sup>S]proIL-1 $\beta$ , except the pH was 6.5. Data for **d** are the average of two independent experiments.



To identify the enzyme responsible for PARP inactivation in mammalian cells during apoptosis, it was purified to homogeneity from cultured human cells. PARP cleavage activity was highly elevated in osteosarcoma cells as they progressed into apoptosis upon reaching confluence; however, owing to the impracticality of obtaining sufficient quantities of an adherent cell line to purify the protease, THP-1 cells were used. Two lines of evidence suggested that the cysteine protease that cleaved PARP in THP-1 cells was the same as that in apoptotic osteosarcoma cells. First, and most compelling, the kinetic parameters for catalysis, and inhibition by Ac-DEVD-CHO, were found to be virtually identical for the enzyme in extracts from both cell types (see below). Second, RT-PCR indicated that, with the exception of ICE (for which transcripts were detectable in THP-1 cells only), both THP-1 cells and apoptotic osteosarcoma cells contained transcripts for the same complement of ICE/CED-3-

**METHODS.** a, [ $^{35}$ S]PARP cleavage was measured in incubation mixtures containing 10  $\mu$ g protein from the cytosol fraction of apoptotic osteosarcoma cells (from 7-day, postconfluent cultures) that were preincubated for 20 min at 37  $^{\circ}$ C in the presence of 100  $\mu$ M 4-amidinophenylmethanesulphonylfluoride (pAPMSF), 2  $\mu$ g ml $^{-1}$  aprotinin, 100  $\mu$ M elastinal, 1 mM phenylmethylsulphonylfluoride (PMSF), 100  $\mu$ M L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone (TLCK), 100  $\mu$ M L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK), 1 mg ml $^{-1}$  soybean trypsin inhibitor (SB-TI), 10  $\mu$ M amastatin, 10  $\mu$ M bestatin, 50  $\mu$ M diprotin A, 8.5  $\mu$ M phosphoramidon, 1  $\mu$ M pepstatin, 5 mM EDTA, 1 mg ml $^{-1}$   $\alpha_2$ -macroglobulin, 100  $\mu$ M antipain, 100  $\mu$ M chymotrypsin, 100  $\mu$ M leupeptin, 10  $\mu$ M E-64, 5 mM iodoacetamide, 5 mM N-ethylmaleimide, 1  $\mu$ M Ac-YVAD-CHO, 100 nM Ac-DEVD-CHO, 100 nM Ac-DEVD COOH or vehicle (lanes 28 to 31). b, The tetrapeptide aldehyde Ac-YVAD-CHO was synthesized as described previously<sup>45</sup>, and Ac-DEVD-CHO (inset) was synthesized essentially by the same procedure. [ $^{35}$ S]PARP cleavage activity was measured in mixtures containing 10  $\mu$ g protein from the cytosol fraction of apoptotic osteosarcoma cells (from 7-day, postconfluent cultures) that were preincubated for 20 min at 37  $^{\circ}$ C with the indicated concentrations of Ac-YVAD-CHO (filled squares) or Ac-DEVD-CHO (open circles) before the addition of [ $^{35}$ S]PARP. Data are the average of two independent experiments. c, Cleavage of [ $^{35}$ S]proIL-1 $\beta$  by ICE and cleavage of [ $^{35}$ S]PARP by the purified PARP cleavage protease (see Fig. 3) was measured as described for Fig. 1c in the presence of varying concentrations of purified recombinant CrmA.





like enzymes (namely, ICE<sub>rel-II</sub>, Nedd-2/ICH-1 and CPP-32 but not ICE<sub>rel-III</sub>) (not shown).

When cytosol fractions from THP-1 cells were resolved by diethylaminoethyl (DEAE) anion-exchange chromatography, the PARP cleavage activity was separated from ICE immunoreactivity and proIL-1 $\beta$  cleavage activity, which coeluted from the column at a lower salt concentration (approximately 80 mM and 100 mM for ICE and PARP cleavage activities, respectively). The fractions containing PARP cleavage activity from several chromatographic runs were pooled and re-chromatographed under identical conditions (Fig. 3a). To selectively purify this activity, two biotinylated derivatives of the Ac-DEVD-CHO tetrapeptide aldehyde inhibitor were synthesized as affinity ligands for the PARP cleavage enzyme (Fig. 3b). Biotinylated affinity ligands were used because they could be pre-incubated with the enzyme to overcome slow ligand binding (see below), by allowing full equilibrium to occur before collection. Both biotinylated tetrapeptide aldehydes had IC<sub>50</sub> values for inhibition of the PARP cleavage enzyme that were comparable to that of the non-biotinylated parent compound (0.2 nM; not shown). The DEAE-chromatography fraction at the peak of PARP cleavage activity was incubated with the biotinylated tetrapeptide aldehydes, then collected with streptavidin-agarose. After extensive washing, the purified PARP cleavage enzyme was eluted from the column with 2 mM biotin. SDS-PAGE (polyacrylamide gel electrophoresis) of the resulting samples indicated that the purified PARP cleavage enzyme was composed of two major polypeptides of approximate  $M_r$  17K and 12K (Fig. 3c).

### Structure of PARP cleavage protease

Electrospray mass spectroscopy analysis of the purified PARP cleavage enzyme indicated that the  $M_r$  of the larger polypeptide was  $16,617.1 \pm 3.1$  and the smaller polypeptide was  $11,896 \pm 1.2$  (Fig. 4a). N-terminal sequence determination and tryptic maps of the purified enzyme identified it as CPP-32, a member of the ICE/CED-3 family of cysteine protease of unknown function which was recently cloned from Jurkat cells<sup>26</sup>. Cloned CPP-32 was originally identified as two isoforms (CPP-32 $\alpha$  and CPP-32 $\beta$ ) which differ by a single conservative amino-acid substitution (Asp 190 versus Glu 190 for CPP-32 $\alpha$  and CPP-32 $\beta$ , respectively). The N-terminal sequence of the 12K subunit of the purified PARP cleavage enzyme corresponded with that of CPP-32 $\beta$  (Fig. 4b), as did the cDNA sequence of human CPP-32 cloned from placenta, lung and kidney (not shown).

Both the mass determination and N-terminal sequence of the two subunits were in agreement, and demonstrated that both polypeptides were derived from the same 32K CPP-32 precursor polypeptide by cleavage between Asp 28-Ser 29 and Asp 175-Ser 176 (Fig. 4b). The organization of the CPP-32 proenzyme is similar to that of ICE (Fig. 4c). Like ICE, the CPP-32 proenzyme is composed of an N-terminal pro-domain followed by a larger subunit (p17) that contains the putative active site cysteine and histidine residues; this is then followed by the smaller subunit (p12). The major differences between ICE and CPP-32 pro-enzymes are that the pro-domain of CPP-32 is substantially shorter, and there is no linker peptide separating the larger (p17) subunit of CPP-32 from the smaller (p12) subunit. The presence of Asp residues in the P<sub>1</sub> position of both the pro-domain/p17 junction and the p17/p12 junction of CPP-32 suggests that autocatalysis plays an important role in pro-enzyme activation, as has been demonstrated for ICE<sup>7,36,37</sup>.

Sequence alignment of all known members of the ICE/CED-3 family of cysteine proteases indicates that CPP-32 is the most closely related of the mammalian enzymes to CED-3, the proapoptotic, nematode cell-death-abnormal *ced-3* gene product (Fig. 4d). An alignment of the five known human enzymes in this family and CED-3 indicates that there is absolute conservation of the residues involved in catalysis, as well as those involved in binding the carboxylate side chain of the substrate P<sub>1</sub> aspartic acid<sup>27,38</sup> (Fig. 4e). Conservation is also high near the Asp-Ser

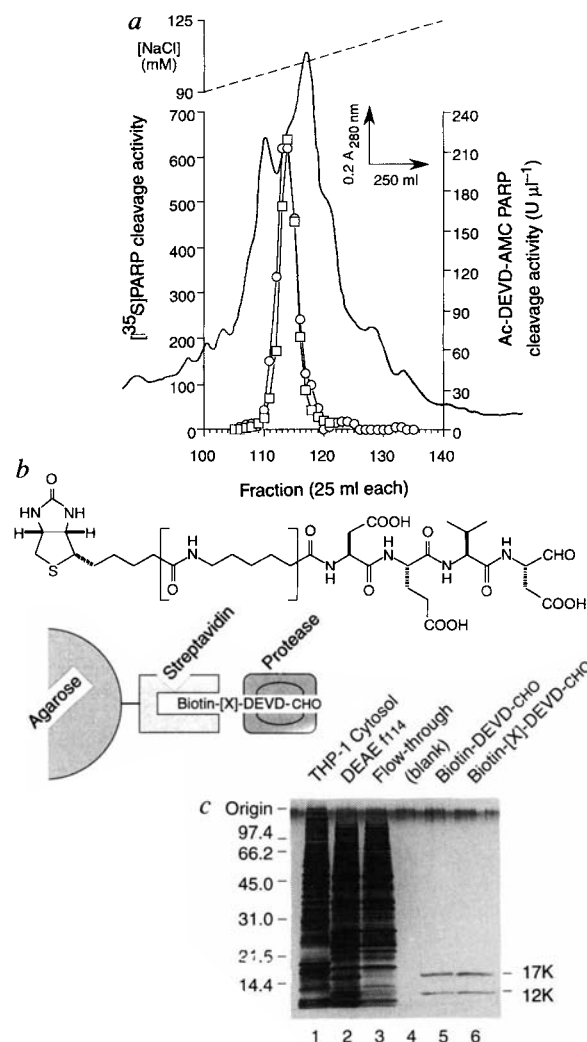
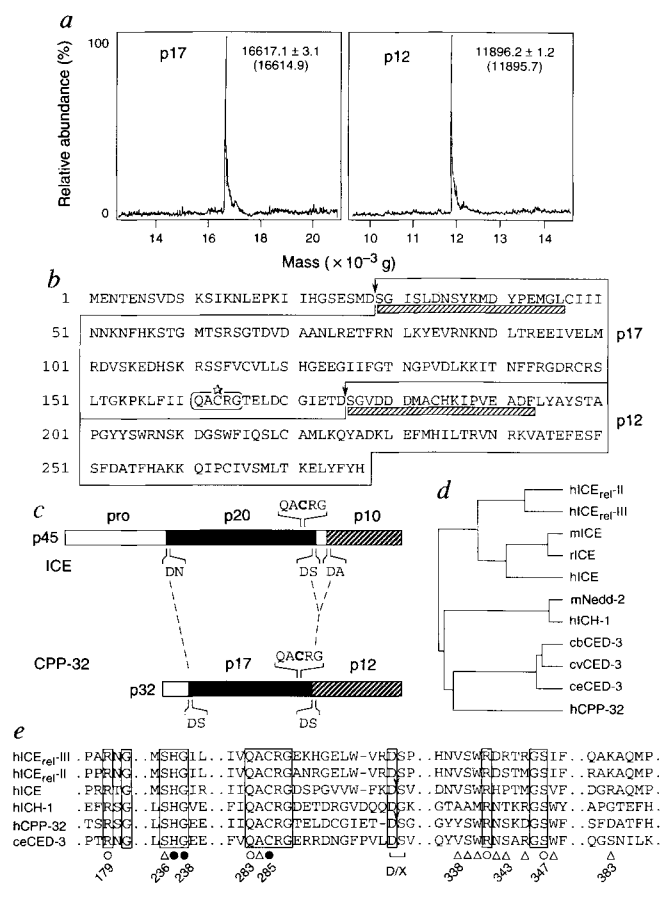


FIG. 3 Purification of the PARP cleavage protease from THP-1 cells. **a**, DEAE anion-exchange chromatography. **b**, Structure of biotinylated tetrapeptide-aldehyde affinity ligands. **c**, SDS-PAGE of THP-1 cell cytosol fraction (lane 1, 9 μg), DEAE peak active fraction (fraction 114) before (lane 2, 6 μg) and after (lane 3, 6 μg) affinity chromatography, eluent from Biotin-DEVD-CHO (lane 5, 0.1 μg) and Biotin-[X]-DEVD-CHO (lane 6, 0.1 μg) affinity columns.

**METHODS.** **a**, The cytosol fraction from cultured THP-1 cells was isolated, dialysed and concentrated then resolved by anion-exchange chromatography as described previously<sup>46</sup>. Fractions corresponding to approximately 90 to 120 mM NaCl, which immediately followed those containing ICE activity, were pooled, and the pools from 25 chromatography runs were combined (1.6 g protein, from  $3.5 \times 10^{12}$  THP-1 cells) and re-run under identical conditions. PARP cleavage activity was measured using [<sup>35</sup>S]PARP or the synthetic fluorogenic tetrapeptide-aminomethylcoumarin (Ac-DEVD-AMC) described in Fig. 5. **b**, Biotin-DEVD-CHO and Biotin-[X]-DEVD-CHO differ by the presence of a 0.9 nm spacer arm (square brackets) which is present in Biotin-[X]-DEVD-CHO but absent in Biotin-DEVD-CHO. These ligands were prepared by: synthesis of *t*-Boc-Asp(OBn)-Glu(OBn)-Val-Asp-CHO protected as the benzylated lactol at the aldehyde; removal of the *t*-Boc group; acylation of the free amine with biotin (for Biotin-DEVD-CHO) or biotinamidocaproic acid (for Biotin-[X]-DEVD-CHO) using EDCI and HOBt. **c**, The fraction from DEAE chromatography corresponding to the peak of PARP cleavage activity (fraction 114; 2.5 ml containing 3 mg protein) was incubated with 20 nmol of Biotin-[X]-DEVD-CHO, then passed through a streptavidin-agarose column (1 ml bed volume; binding capacity, 63 nmol biotin ml<sup>-1</sup>) and washed. The enzyme was eluted by perfusing the column with 2 mM α-biotin. (Biotin-DEVD-CHO yielded comparable results.) Samples were resolved on 14% SDS/polyacrylamide gels, and protein bands were visualized by silver staining.

**FIG. 4** Structure of PARP cleavage protease; CPP-32. **a**, Electrospray mass spectroscopy analysis of 17K (left) and 12K (right) subunits of the purified PARP cleavage protease. Numbers in brackets indicate the calculated masses of the subunits based on the sequences described in **b**. **b**, Primary structure of CPP-32. The deduced amino-acid sequence from the CPP-32 $\beta$  cDNA clone<sup>26</sup> is shown. Hatched bars indicate the N-terminal sequences determined for the purified enzyme subunits. Arrowheads mark the Asp 28–Ser 29 and Asp 175–Ser 176 cleavage sites which yield the p17 and p12 subunits from the CPP-32 pro-enzyme. **c**, Comparison of ICE and CPP-32 pro-enzyme organization. **d**, Phylogenetic relationship of all known members of the ICE/CED-3 family of cysteine protease. **e**, Multiple sequence alignment of all known human ICE/CED-3-like proteases (top 5 sequences) and nematode CED-3 (bottom sequence). Numbering corresponds to the residue position within human ICE. Regions implicated in substrate binding to human ICE, based on the X-ray crystal structure<sup>37,38</sup>, are shown: filled circles, catalytic; open circles, binding pocket for carboxylate of P<sub>1</sub> Asp; open triangles, proximity (<0.4 nm) to P<sub>2</sub>–P<sub>4</sub> residues. Arrowheads indicate known pro-enzyme cleavage sites for ICE and CPP-32.

**METHODS.** **a**, Portions of the purified PARP cleavage enzyme described in Fig. 3c were resolved on a narrow-bore C4 reverse-phase HPLC column, and the individual subunits were analysed by capillary liquid chromatography coupled to a triple-sector quadrupole mass spectrometer equipped with an electrospray ion source, essentially as described previously<sup>7</sup>. Tryptic peptides were also generated from the purified subunits and analysed by electrospray mass spectroscopy to further substantiate identification as CPP-32. Peptides and their respective predicted and observed masses were: IPVEADFLYAYSTAPGYYSWR-207, 2470.8, 2470.2; VATEFESFSFDTAFHAK-259, 1935.1, 1934.7; LEFMHILTR-238, 1160.4, 1161.0; ELYFYH-277, 872.0, 872.0. **b**, Approximately 100 pmol of the purified PARP cleavage enzyme described in Fig. 3c was resolved on a 14% SDS/polyacrylamide gel and transferred to a polyvinylidenedifluoride membrane for N-terminal sequence determination of the individual p17 and p12 subunits (hatched bars). **d**, **e**, Deduced polypeptide sequences (entire open reading frame) for the indicated cDNA or gene sequences were aligned using the Genetics Computer Group (Madison WI) PILEUP algorithm<sup>47</sup> (gap weight, 3.0; gap-length weight, 0.1) and are presented as a phylogenetic dendrogram (**d**) and an amino-acid alignment (**e**).



cleavage site at the C terminus of the p20 subunit of ICE (Asp 296–Ser 297) and the p17 of CPP-32 (Asp 175–Ser 176), suggesting that the active forms of the other members of this family are also heterodimers derived from pro-enzymes. Residues that might form the P<sub>2</sub>–P<sub>4</sub> binding pockets, however, are not widely conserved, indicating that substrate specificity might be determined by one or more of these amino acids.

### Kinetic properties of CPP-32

A continuous fluorometric assay for CPP-32 was developed with the substrate Ac-DEVD-AMC (amino-4-methylcoumarin). The design of this substrate was based on the tetrapeptide-AMC motif that has been used successfully with ICE<sup>7</sup>, using the PARP cleavage site P<sub>1</sub>–P<sub>4</sub> tetrapeptide (Fig. 5a inset). Cleavage of this substrate by CPP-32 showed Michaelis–Menton kinetics with a  $K_m$  value of  $9.7 \pm 1.0$   $\mu$ M (Fig. 5a). This assay has facilitated a detailed investigation of the mechanism of inhibition of the enzyme by the tetrapeptide aldehyde Ac-DEVD-CHO.

Peptide aldehydes are potent, reversible inhibitors of cysteine proteases that undergo nucleophilic addition of the catalytic cysteine to form a thiohemiacetal. Although the potency of aldehyde inhibitors was originally attributed to their ability to mimic the transition state in amide bond hydrolysis<sup>39</sup>, the recently determined crystal structure of ICE with the tetrapeptide aldehyde Ac-YVAD-CHO clearly shows this inhibitor bound in a non-transition-state conformation, with the oxyanion of the thiohemiacetal being stabilized by the active site histidine<sup>37</sup>. The tetrapeptide aldehyde containing the appropriate recognition sequence for CPP-32, Ac-DEVD-CHO, is a potent, competitive inhibitor of this enzyme. It is slow binding, as shown by the time-dependent approach to equilibrium observed when enzyme was added to reaction mixtures containing inhibitor (50 nM) and  $1 \times K_m$  substrate (Fig. 5b). The solid line of Fig.

5b (filled circles) is theoretical for an association rate constant  $k_{on} = 1.3 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>. This rate constant, which is well below theoretical predictions of rates for diffusion-limited reactions ( $10^8$  to  $10^{10}$  M<sup>-1</sup> s<sup>-1</sup>), is similar to the corresponding rate constant for association of ICE with its tetrapeptide aldehyde inhibitor ( $k_{on}$  Ac-YVAD-CHO =  $3.8 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>). The nearly complete suppression of the activity that was seen at effectively infinite time using 50 nM Ac-DEVD-CHO defines a  $K_i$  for inhibition of CPP-32 of <1 nM, making this among the most potent peptide aldehydes known for a cysteine protease.

In contrast to the potent inhibition of CPP-32 observed with the tetrapeptide aldehyde Ac-DEVD-CHO, the ICE inhibitor Ac-YVAD-CHO ( $K_{iICE} = 0.76$  nM) is a very weak inhibitor of CPP-32 ( $K_{iCPP-32} = 12$   $\mu$ M), indicating that these enzymes have distinct peptide substrate specificities. This differential in potency can be attributed to the fact that residues implicated in binding the P<sub>4</sub> Tyr of proIL-1 $\beta$ , which is a key determinant for ICE, are not conserved in CPP-32. The crystal structure of active ICE, for example, indicates that the two key amino acids that interact with the P<sub>4</sub> Tyr of proIL-1 $\beta$  are His 342 and Pro 343, which are replaced by Asn and Ser, respectively, in both CPP-32 and CED-3 (Fig. 4e). These latter residues in CPP-32 would be better able to form the hydrogen bonds necessary to interact with the carboxylate side chain of the P<sub>4</sub> Asp of PARP. The enzymes also clearly have different macromolecular substrate specificities: purified ICE was unable to cleave PARP, and purified CPP-32 did not cleave proIL-1 $\beta$  at either the FEAD 27–G 28 or DEVD 216–G 217 cleavage sites (a 5,000-fold excess of each enzyme was tested; not shown). The enzymes are also distinguished by their behaviour with the cowpox serpin, CrmA, which shows a 10,000-fold preference for ICE.

The catalytic and inhibitor constants for the PARP cleavage activity in extracts of THP-1 cells, apoptotic osteosarcoma cells,

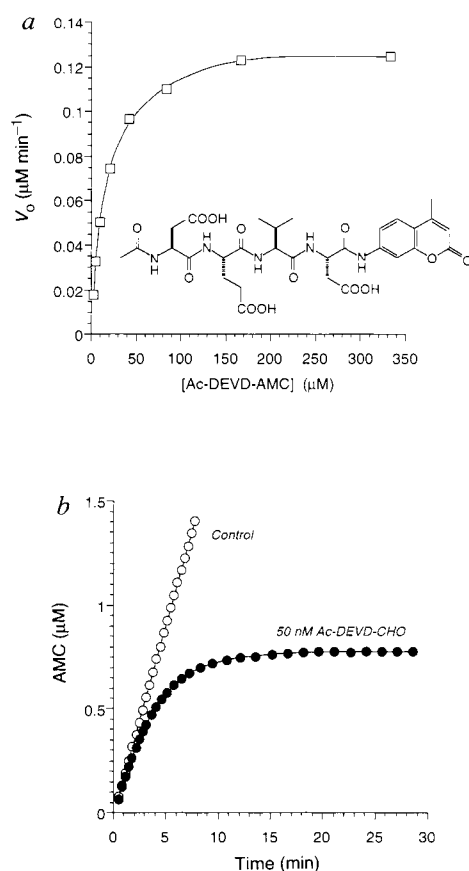


FIG. 5 Kinetic parameters for CPP-32 catalysis and inhibition. **a**, Determination of  $K_m$  for Ac-DEVD-AMC (structure in insert). **b**, Kinetics of inhibition of CPP-32 by the peptide aldehyde Ac-DEVD-CHO. **c**, Comparison of PARP cleavage activity and inhibition by Ac-DEVD-CHO in THP-1 cell, osteosarcoma cell and chicken S/M extracts.

**METHODS.** **a**, Ac-DEVD-AMC (inset) was prepared as follows: synthesis of *N*-Ac-Asp(OBn)-Glu(OBn)-Val-CO<sub>2</sub>H; coupling with Asp(OBn)-7-AMC; removal of benzyl groups. Reaction mixtures, which contained the indicated concentrations of Ac-DEVD-AMC and 100 U ml<sup>-1</sup> of the purified PARP-cleavage CPP-32 enzyme (1 U represents 1 pmol AMC liberated per min at 25 °C at saturating substrate concentration), were monitored continuously in a spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Initial velocities and substrate concentrations were fit by nonlinear regression to the Michaelis-Menten equation (solid line). **b**, Reactions contained 1 ×  $K_m$  Ac-DEVD-AMC (10  $\mu\text{M}$ ) and the purified PARP-cleavage CPP-32 enzyme (400 U ml<sup>-1</sup>). Addition of the tetrapeptide aldehyde (50 nM) to this reaction mixture resulted in a time-dependent loss of enzyme activity (filled circles) whereas the reaction not containing inhibitor was strictly linear (open circles). The association rate constant ( $k_{on}$ ) was calculated from several progress curves according to equations for slow and tight-binding inhibitors<sup>48</sup>. The complete inhibition of activity observed at infinite time with 50 nM Ac-DEVD-CHO indicates a  $K_i$  of less than 1 nM for this inhibitor. **c**, Extracts from THP-1 cells, apoptotic osteosarcoma cells and chicken DU249 cells committed to apoptosis were prepared as described for Fig. 1. The  $K_m$  for cleavage of the synthetic fluorogenic tetrapeptide Ac-DEVD-AMC and the  $k_{on}$  and  $K_i$  values for the tetrapeptide aldehyde inhibitor Ac-DEVD-CHO were determined as described above.

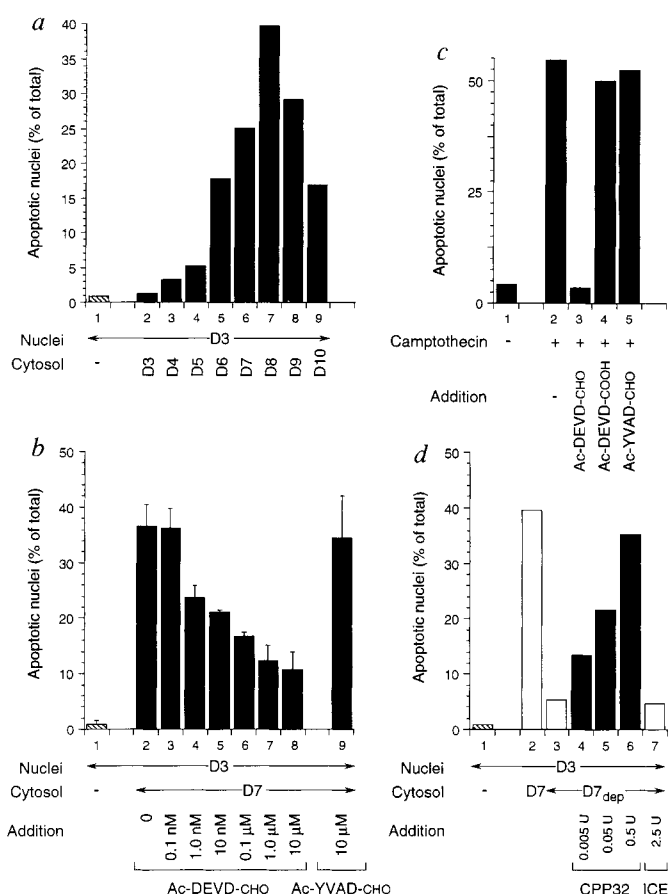


FIG. 6 *In vitro* apoptosis and selective inhibition by Ac-DEVD-CHO or by depletion of CPP-32-mediated PARP cleavage activity. **a**, Cytosols from progressively apoptotic osteosarcoma cells confer apoptotic changes upon healthy nuclei from non-apoptotic cells. **b**, **d**, Attenuation of *in vitro* apoptosis by inhibition or depletion of CPP-32. **c**, Inhibition of camptothecin-induced apoptosis of osteosarcoma cells by Ac-DEVD-CHO.

**METHODS.** Nuclei were isolated from non-apoptotic (day 3) cells essentially as described before<sup>41</sup>, except that the nuclear isolation buffer was 10 mM PIPES/KOH (pH 7.4), 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10  $\mu\text{M}$  cytochalasin B, 1 mM phenylmethylsulphonyl fluoride, 10  $\mu\text{g ml}^{-1}$  pepstatin A, 20  $\mu\text{g ml}^{-1}$  leupeptin, 10  $\mu\text{g ml}^{-1}$  aprotinin. **a**, The isolated nuclei from  $2 \times 10^6$  day-3 cells were combined with 25  $\mu\text{l}$  of the cytosol fraction ( $2.5 \times 10^6$  cell equivalents) from cells maintained for the indicated times in culture (see Fig. 1) then incubated in 100  $\mu\text{l}$  (final volume) of a mixture containing 10 mM HEPES/KOH (pH 7.0), 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 40 mM  $\beta$ -glycerophosphate, 1 mM dithiothreitol, 2 mM ATP, 10 mM creatine phosphate and 50  $\mu\text{g ml}^{-1}$  creatine kinase. After 2 h at 37 °C, nuclear chromatin was stained with 5  $\mu\text{g ml}^{-1}$  Hoechst 33342 and examined by fluorescent microscopy. For each condition, a minimum of 250 nuclei in 5 separate fields were scored. Data are the average of 2 independent experiments. **b**, *In vitro* apoptosis was measured as described in **a** using nuclei from non-apoptotic, day-3 osteosarcoma cells (1–9) combined with the cytosol fraction from apoptotic, day 7 cells (2–9) in the presence of the indicated concentrations of Ac-DEVD-CHO (3–8) or Ac-YVAD-CHO (9). Data are the average of 3 independent experiments  $\pm$  s.e.m. **c**, Intact day-3 osteosarcoma cell cultures were preincubated for 60 min at 37 °C with vehicle (2) or with 100  $\mu\text{M}$  Ac-DEVD-CHO (3), Ac-DEVD-COOH (4) or Ac-YVAD-CHO (5), then treated with 1  $\mu\text{g ml}^{-1}$  camptothecin (2–5) for an additional 4 h to induce apoptosis. Apoptosis was assessed by fluorescent microscopy as described in **a**. **d**, PARP cleavage activity was depleted from the cytosol fraction of apoptotic, day-7 cells (D7<sub>dep</sub>) by incubating 1 ml of the fraction with 100 nM Biotin-[X]-DEVD-CHO for 20 min then collecting with streptavidin-agarose. *In vitro* apoptosis was measured as described in **a**, using nuclei from non-apoptotic, day-3 osteosarcoma cells (1–7) combined with untreated day-7 cytosol (2) or depleted day-7 cytosol (3–7) supplemented with varying concentrations of purified CPP-32 (4–6) or purified ICE (7).

and apoptotic chicken S/M extracts were virtually identical (Fig. 5c), strongly suggesting that the same enzyme (CPP-32) cleaves PARP in all these cell types.

### Attenuation of apoptosis

Apoptotic events can be re-constituted *in vitro*. The isolated nuclei from healthy cells undergo the morphological changes that are characteristic of apoptosis (for example, chromatin condensation, fragmentation and margination, as well as internucleosomal DNA cleavage) when they are incubated with the cytosol fraction from apoptotic cells<sup>40,41</sup>. Because the most potent and selective inhibitor of CPP-32-mediated PARP cleavage (Ac-DEVD-CHO) had poor membrane permeability, this system was established with human cells and used to study the effects of CPP-32 inhibition or depletion on apoptosis *in vitro*. Cytosols from non-apoptotic osteosarcoma cells had little effect on nuclear morphology, whereas those from progressively apoptotic cells were capable of inducing apoptosis-like changes in the recipient nuclei (Fig. 6a). The degree of apoptotic morphology conferred upon the otherwise healthy nuclei coincided with the degree of apoptosis occurring in the cells from which the cytosols were extracted (see Fig. 1c) as well as the level of PARP cleavage activity (see Fig. 1d).

If the PARP-cleaving CPP-32 cysteine protease is important for apoptosis then inhibition or depletion of its activity should prevent these nuclear changes from occurring. Morphological changes that occurred when the cytosol fraction from apoptotic osteosarcoma cells was incubated with healthy nuclei from non-apoptotic cells could be attenuated by the tetrapeptide aldehyde inhibitor of CPP-32-mediated PARP cleavage, Ac-DEVD-CHO ( $IC_{50}$  = 10 to 100 nM), but not with the ICE inhibitor, Ac-YVAD-CHO (Fig. 6b). The camptothecin-stimulated apoptotic death of intact osteosarcoma cells was also prevented by Ac-DEVD-CHO (albeit at high concentrations only (100  $\mu$ M)), whereas neither the inactive carboxylic acid (Ac-DEVD-COOH) nor the ICE inhibitor (Ac-YVAD-CHO) had any effect (Fig. 6c). Furthermore, if CPP-32 were depleted from the cytosol fraction of apoptotic osteosarcoma cells using the biotinylated affinity ligand, these PARP cleavage-deficient extracts were

largely incapable of conferring apoptotic changes to healthy recipient nuclei (Fig. 6d). The pro-apoptotic capabilities of depleted extracts were restored when they were supplemented with purified CPP-32, but not when they were supplemented with purified ICE. In the absence of a cytosolic fraction, CPP-32 alone did not provoke apoptotic changes to healthy nuclei (not shown), demonstrating that CPP-32 is necessary but not sufficient for apoptosis. Together, these experiments suggest that CPP-32 initiates key events in apoptosis and that inhibition or depletion of its activity prevents apoptosis from occurring.

### Conclusions

In the nematode *C. elegans*, deletion or mutation of a single gene, *ced-3*, abolishes apoptotic death<sup>5</sup>. When sequenced, *ced-3* was found to be homologous to the gene for mammalian interleukin-1 $\beta$ -converting enzyme (ICE)<sup>6</sup>, which encodes a protease whose only known function is the cleavage of the inactive 31K proIL-1 $\beta$  cytokine precursor to the active 17K form. How the apoptotic role of an ICE-like protease in mammalian cells can be accounted for, given the commitment of ICE to IL-1 $\beta$  formation, and the finding that apoptosis occurs normally in ICE-deficient mice<sup>29,30</sup>, has become more obvious with the discovery of four other mammalian ICE/CED-3-like proteases (ICE<sub>rel</sub>-II, ICE<sub>rel</sub>-III, Nedd-2/ICH-1 and CPP-32)<sup>24-27</sup> and the observation that PARP, an enzyme involved in the coordination of genome structure and integrity in stressed cells, is functionally inactivated by a protease resembling ICE (prICE) at the onset of apoptosis<sup>30</sup>. We have demonstrated that prICE is in fact CPP-32, and that CPP-32 is the specific ICE/CED-3-like cysteine protease that cleaves PARP in mammalian cells. The name apopain is proposed for the active form of this enzyme to replace prICE and CPP-32. The central role played by CPP-32/apopain in mammalian cell death is further substantiated by potent and selective inhibitors that prevent apoptosis from occurring *in vitro* and *in vivo*. These findings, together with the sequence relationship between CPP-32 and CED-3, suggests that CPP-32 may be the human equivalent of CED-3. The pharmacological modulation of CPP-32/apopain activity may therefore be an appropriate point for therapeutic intervention in pathological conditions where inappropriate apoptosis is prominent.  $\square$

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# Imaging of transport currents in superconducting $(\text{Bi, Pb})_2\text{Sr}_2\text{Ca}_2\text{Cu}_3\text{O}_x$ composites

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IMPROVEMENTS in the current carrying capacity of high- $T_c$  superconducting composite conductors will come from a detailed understanding of the connection between current flow and microstructure. Slicing experiments<sup>1-3</sup> on silver-sheathed monofilamentary  $(\text{Bi, Pb})_2\text{Sr}_2\text{Ca}_2\text{Cu}_3\text{O}_x$  (Bi-2223), combined with microstructural studies<sup>4,5</sup>, have suggested that current flow is enhanced in well textured Bi-2223 layers at the superconductor/silver interface, but the spatial resolution of the slicing experiments is insufficient to identify the actual current path. More recently, magneto-optical imaging in an applied field<sup>6-10</sup> has been used to measure spatial variations in the shielding critical current density on a micrometre scale in Bi-2212 and Bi-2223 mono- and multifilamentary composites. Here we use an extension of this technique to measure spatial variations in transport critical current density,  $J_c$ , in a Bi-2223 multifilamentary composite at close to real operating conditions. Current densities of up to  $8 \times 10^4 \text{ A cm}^{-2}$  at 77 K in self-field are observed in well aligned Bi-2223 grain colonies of 2–3  $\mu\text{m}$  width. The superconductor/silver interface does not, in general, constitute a continuous high-current path because of

frequent interruptions by second-phase particles; elimination of these particles in the interface layer could result in a threefold increase in  $J_c$ .

Magneto-optical flux imaging has been widely used to study superconductors in the presence of magnetic fields<sup>6-10</sup>. The applied field induces superconducting shielding currents which flow in closed loops and are concentrated in regions of strong flux pinning. Thus, images obtained in this way directly identify regions of high/low intragranular critical current density. In studies on Bi-2223 composites<sup>9,10</sup>, a large variation of intragranular critical current densities was observed with the highest values often occurring near the superconductor/silver interface. These studies do not, however, reveal the nature of transport current flow, which is largely determined by the intergranular critical current density, that is, the connectivity between superconducting grains. A grain with very high intragranular critical current density will carry no transport current if it is poorly coupled to the neighbouring grains. The transport current path is a result of a percolation process among all the superconducting grains. Here we show that this path of a transport current in multifilamentary Bi-2223 can be determined quantitatively by imaging its magnetic patterns.

The sample is a silver-sheathed Bi-2223 composite conductor fabricated using the 'oxide powder in tube' process<sup>11,12</sup>. The composite contains 37 filaments, and has a critical current of 31 A corresponding to  $J_c \approx 17,000 \text{ A cm}^{-2}$  at 77 K in self-field. A section ~1.5 cm long was polished to about half its original width, giving a final overall cross-section of  $200 \times 1,370 \mu\text{m}^2$ . Currents up to 21 A were imaged. To avoid excessive heating the current was pulsed, with 'on' times ranging from 0.2 to 0.7 s. The current pulses were synchronized with the exposure of a high-resolution CCD (charge-coupled device) camera and a storage oscilloscope which recorded the voltage developed in the wire. The geometry of the experiment is shown in Fig. 2. The surface being imaged is the  $x$ - $y$  plane while the current is passed along the  $y$ -direction. The sample was polished to expose a stack of four filaments. The electron microscope image of the sample is shown in Fig. 1a. Light grey represents the silver matrix and dark grey the

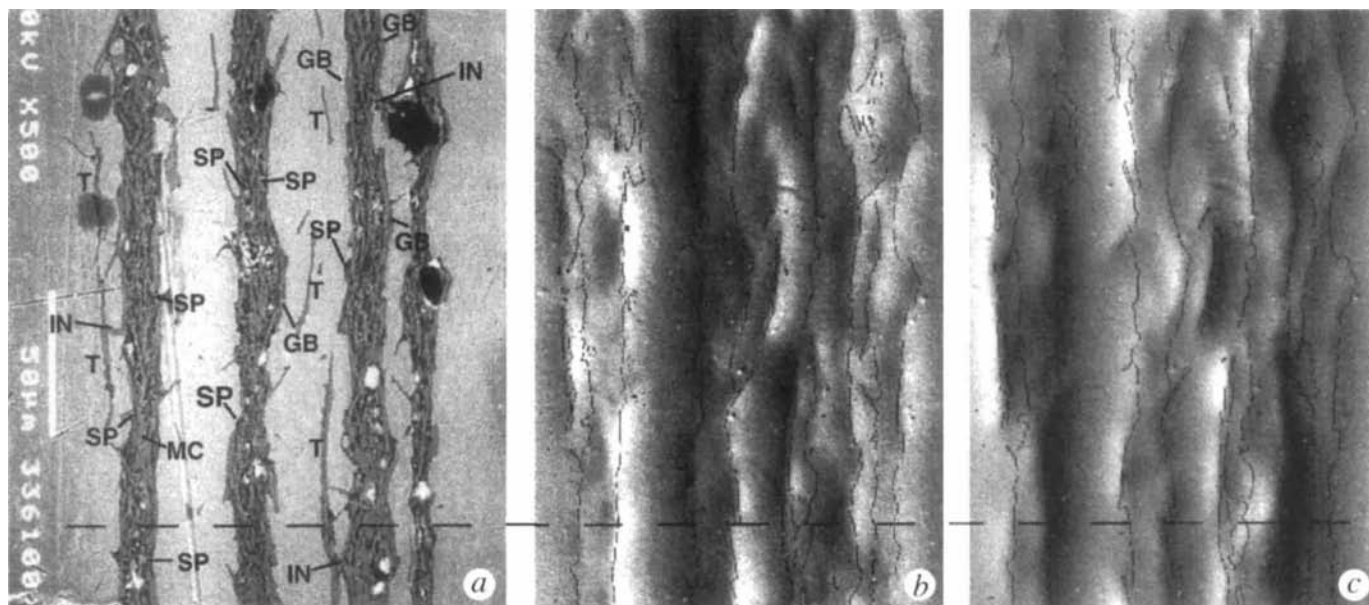


FIG. 1 a, Electron microscope image of the sample. Light grey represents the silver matrix, dark grey Bi-2223. The black and white inclusions are second-phase particles. 'MC' marks a misoriented grain colony, 'T' the tips of filaments in the next stack down, 'SP' second-phase particles discussed in the text, 'IN' interconnections between filaments, and 'GB' grain boundaries between Bi-2223 colonies which show up as kinks between otherwise straight Bi-2223 grains. Scale bar, 50  $\mu\text{m}$ . b and c, magnetic field pattern at 30 K caused by a transport

current of +21 A (b) and -21 A (c). To show the field variations around the filaments and avoid saturation at the right (b) and left (c) edges of the images, linear backgrounds have been subtracted. Therefore, bright and dark levels do not correspond directly to the absolute field values shown in Fig. 2a but rather represent the relative positive and negative fields around the filaments. The dotted lines mark the outlines of the superconducting filaments shown in a. The horizontal dashed line is the position of the field and current profiles shown in Fig. 2.

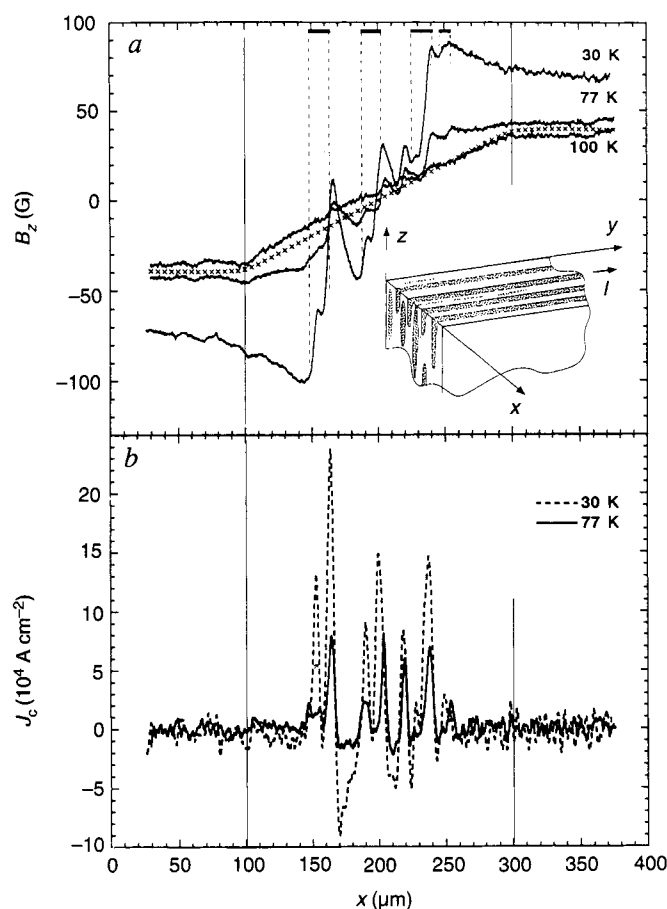


FIG. 2 a, Field profiles due to a transport current of 21 A at 30 K, 77 K and 100 K along the horizontal line in Fig. 1. The crosses represent the calculated pattern for a uniform current of 21 A flowing through a cross-section of  $200 \times 1,370 \mu\text{m}^2$ . The solid vertical lines mark the outside edges of the silver matrix, and the horizontal black bars the positions of the superconducting filaments. The inset shows the geometry of the experiment and indicates the positions of the filaments with respect to the imaged surface. b, Profiles of the current density at 30 K and 77 K. The concentration of current flow near the edges of the filaments is clearly seen. At 77 K, current densities of  $\sim 8 \times 10^4 \text{ A cm}^{-2}$  are observed in the phase-pure well aligned Bi-2223 regions.

Bi-2223, whereas the black and white spots are second-phase particles, mostly alkaline-earth copper oxides. The narrow isolated Bi-2223 segments marked 'T' are the tops of filaments in the next stack down. This has been verified by polishing deeper into the conductor.

Figure 1b, c shows the magnetic field patterns caused by a current of +21 A and -21 A, respectively, applied at 30 K. The patterns are characterized by a sequence of bright and dark contrasts mostly concentrated near the edges of the filaments. Bright represents positive field levels, and dark shows negative field levels (see Fig. 1 legend). On changing the direction of the transport current, the bright/dark sequences reverse.

Figure 2a shows field profiles taken along the dashed line indicated in Fig. 1. The field profiles at 30 K and 21 A for the first and second filaments are characterized by an intermediate maximum in the normal component of the magnetic induction,  $B_z(x)$ , near the centre of the filament. This indicates a non-uniform current distribution consisting of two current paths parallel to each other. At 77 K the applied current of 21 A is above the critical current for the polished composite as evidenced by the appearance of a voltage across the sample. The corresponding field patterns show reduced modulations of the field above the filaments, indicating that less current is flowing in the filaments. The overall structure of the field profile, however, does not change when the current goes from subcritical to supercritical as the temperature is increased. In addition, at 77 K a slope discontinuity in  $B_z(x)$  occurs near  $100 \mu\text{m}$  and  $300 \mu\text{m}$ , the positions of the outside edges of the silver matrix. This indicates current sharing between the superconducting filaments and the silver matrix. At 100 K the current flows essentially uniformly through the entire conductor, as shown by the good agreement of the measured field profile with the calculated profile. The calculation is based on the analytical expression<sup>13</sup> for the magnetic field above a rectangular conductor.

For conductors whose thicknesses,  $t$ , measured along the  $z$  direction, are larger than their widths,  $w$ , measured along  $x$ , the field gradients above the conductor are given by  $\partial B_z / \partial x = -(\mu_0 J_y / 2) (1 - w / \pi t)$  in terms of the current density along the  $y$ -direction,  $J_y$ , which can be derived from ref. 13. Grain colonies in the Bi-2223 composites generally have aspect ratios  $t/w \gg 1$  (refs 4, 5, 10). Thus, the current density distribution can be obtained from a derivative of the field maps as shown in Fig. 3a. Here, white and red areas represent high current density. A comparison with Fig. 1a shows that high current densities are observed in thin layers ( $2\text{--}3 \mu\text{m}$  width) of well aligned phase-pure Bi-2223 grain colonies, which account for roughly 10–15% of the total superconducting area. In the majority of cases these are located close to the silver. However, a continuous current path along the interface is developed only on the right-hand edge of the third filament. For all the other filaments, this current path is frequently interrupted by second-phase particles (marked 'SP'). This is demonstrated in Fig. 3b and c, which shows contours of the current density overlaid on the microstructure of the selected areas indicated in Fig. 3a. The continuous current path on the right-hand edge of the left-hand filament in Fig. 3c and its correspondence to an uninterrupted, although meandering, layer of Bi-2223 is clearly seen. In contrast, the right-hand edge of the filament in Fig. 3b directly demonstrates the disruptive effect of several small second-phase particles located in the interface layer. Thus, the beneficial effect of the enhanced interface area which might have been expected from the studies on monofilaments<sup>1–3</sup> is largely offset by the detrimental effect of second-phase particles which, owing to the narrow width of the interface layer, are very effective in interrupting the current flow along the interface. It is also seen in Fig. 3b and c that the mere presence of well textured Bi-2223 grains at the silver interface does not imply the ability to carry high transport current, as evidenced by the sections labelled 'NC' which carry very little

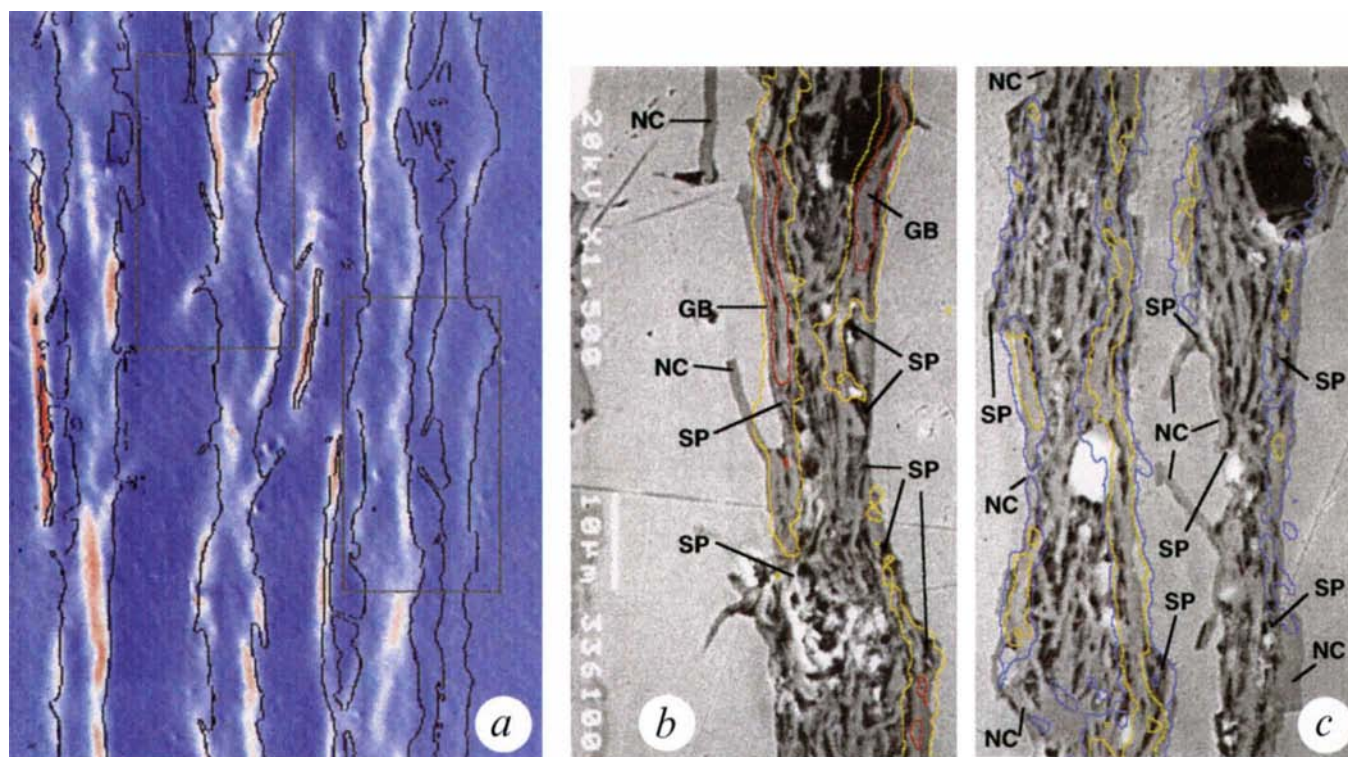


FIG. 3 a, Map of the current flow in the superconducting filaments. White and red represent high current density. This pattern is obtained by taking the x-derivative of the field maps. Current flow is restricted to narrow regions mostly along the edges of the filaments. A comparison with Fig. 1a identifies these regions as phase-pure well aligned Bi-2223. The apparent current flow in isolated regions (marked 'I' in Fig. 1a) or outside the superconductor (between the second and third filaments) is caused by the next stack of filaments just under the exposed surface

or no current at all. These sections are either isolated outgrowths, or are disconnected from a continuous current path due to the positioning of second-phase particles. Small-angle grain boundaries between Bi-2223 grain colonies (misalignment angle  $\theta \approx 15^\circ$ , see positions marked 'GB' in Figs 1a and 3b) do not affect the current flow significantly. Current flow from one edge of a filament to the other occurs along misaligned Bi-2223 grain colonies (for example, position marked 'MC' in Fig. 1a).

Profiles of the current density at 30 K and 77 K are shown in Fig. 2b. The large values at the interfaces are clearly seen. Even at 77 K values of  $\sim 8 \times 10^4 \text{ A cm}^{-2}$  are observed. The numerical uncertainties caused by the unknown aspect ratios of the current-carrying grain colonies are  $<25\%$ . The above expression for the transport current in terms of the field gradient is not valid for the space between the current-carrying grain colonies; thus the negative values for  $J_c$  in Fig. 2b should be ignored.

In the present sample four interconnections between filaments (marked 'IN' in Fig. 1a) are observed, only one of which is found to contribute to current transport. The coupling between filaments is an important parameter for minimizing losses in composite conductors carrying alternating current<sup>14</sup>. Clearly, magneto-optical imaging of transport currents is the ideal way to investigate filament coupling in composites with large filament numbers where interconnections are frequent. □

as discussed above. b and c, microstructure of the areas indicated by boxes in a. Overlaid are contour lines of the current density at levels of  $1.4 \times 10^5$  (red),  $0.8 \times 10^5$  (yellow) and  $0.4 \times 10^5 \text{ A cm}^{-2}$  (blue). Second-phase particles (marked 'SP') cause frequent interruptions of the current path, whereas small-angle grain boundaries ('GB') do not degrade current flow. Well textured Bi-2223 grain colonies that carry only little current or no current at all are marked as 'NC'. Scale bar, 10  $\mu\text{m}$ .

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## Scaling behaviour in the dynamics of an economic index

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THE large-scale dynamical properties of some physical systems depend on the dynamical evolution of a large number of nonlinearly coupled subsystems. Examples include systems that exhibit self-organized criticality<sup>1</sup> and turbulence<sup>2,3</sup>. Such systems tend to exhibit spatial and temporal scaling behaviour—power-law behaviour of a particular observable. Scaling is found in a wide range of systems, from geophysical<sup>4</sup> to biological<sup>5</sup>. Here we explore the possibility that scaling phenomena occur in economic systems—especially when the economic system is one subject to precise rules,

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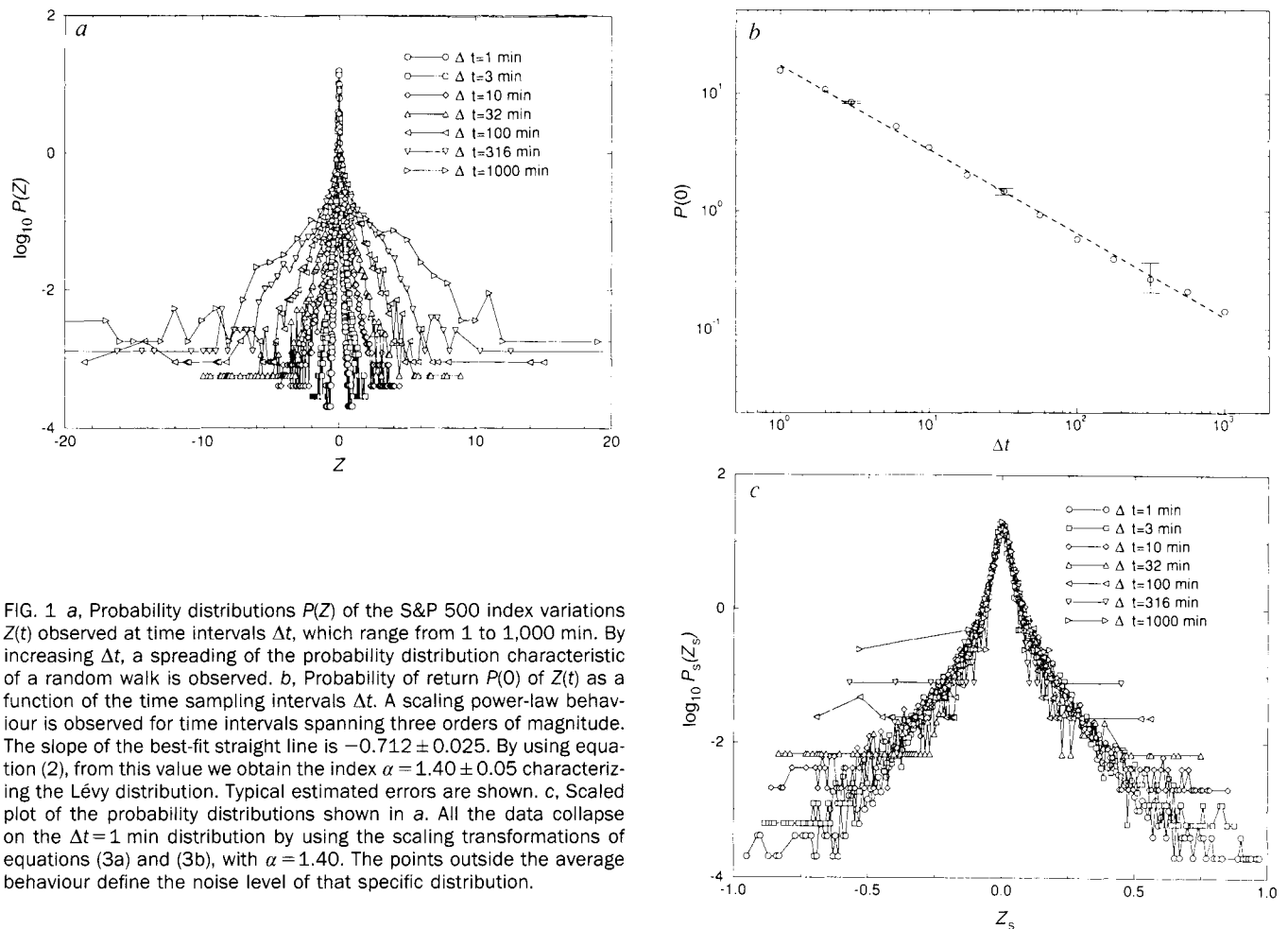


FIG. 1 *a*, Probability distributions  $P(Z)$  of the S&P 500 index variations  $Z(t)$  observed at time intervals  $\Delta t$ , which range from 1 to 1,000 min. By increasing  $\Delta t$ , a spreading of the probability distribution characteristic of a random walk is observed. *b*, Probability of return  $P(0)$  of  $Z(t)$  as a function of the time sampling intervals  $\Delta t$ . A scaling power-law behaviour is observed for time intervals spanning three orders of magnitude. The slope of the best-fit straight line is  $-0.712 \pm 0.025$ . By using equation (2), from this value we obtain the index  $\alpha = 1.40 \pm 0.05$  characterizing the Lévy distribution. Typical estimated errors are shown. *c*, Scaled plot of the probability distributions shown in *a*. All the data collapse on the  $\Delta t = 1$  min distribution by using the scaling transformations of equations (3a) and (3b), with  $\alpha = 1.40$ . The points outside the average behaviour define the noise level of that specific distribution.

as is the case in financial markets<sup>6-8</sup>. Specifically, we show that the scaling of the probability distribution of a particular economic index—the Standard & Poor's 500—can be described by a non-gaussian process with dynamics that, for the central part of the distribution, correspond to that predicted for a Lévy stable process<sup>9-11</sup>. Scaling behaviour is observed for time intervals spanning three orders of magnitude, from 1,000 min to 1 min, the latter being close to the minimum time necessary to perform a trading transaction in a financial market. In the tails of the distribution the fall-off deviates from that for a Lévy stable process and is approximately exponential, ensuring that (as one would expect for a price difference distribution) the variance of the distribution is finite. The scaling exponent is remarkably constant over the six-year period (1984–89) of our data. This dynamical behaviour of the economic index should provide a framework within which to develop economic models.

A problem of interest for both practical and theoretical reasons concerns the distribution of the variations of share price and the dynamical evolution of this distribution. The most widely accepted models state that the variation of share price is a random process. For the distribution of the index returns (which are the difference between two successive logarithms of price), principal proposals include: (1) a normal distribution<sup>12,13</sup>, (2) a Lévy stable distribution<sup>14,15</sup>, and (3) leptokurtic distributions generated by a mixture of distributions<sup>16</sup>, or (4) by ARCH/GARCH models<sup>17,18</sup>. The most obvious difference between the different models involves the wings of the distribution. Distinguishing between the processes by comparing the distribution wings can be quite difficult because data sets are limited. Indeed, different conclusions about the distribution of price variations have been published<sup>12-15,19-24</sup>. In our analysis, we investigate both price difference and returns, and we find the two stochastic

processes have quite similar statistical properties in the high-frequency regime.

We have undertaken a statistical study of timescales as short as 1 min, a value close to the minimum time needed to perform a transaction in the market. Specifically, we investigate the dynamics of a price index of one of the largest financial markets in the world: the New York Stock Exchange. We study the Standard & Poor's 500 index during the six-year period from January 1984 to December 1989, and we find that the S&P 500 is a stochastic process remarkably well described by a Lévy stable symmetrical process<sup>25</sup> except for the most rare events. For a time interval spanning three orders of magnitude, the dynamics of the central part of the distribution are in agreement with the predictions of a Lévy stable process.

Data were obtained from the Chicago Mercantile Exchange. They consist of all 1,447,514 records of the S&P 500 cash index during the period studied. The time intervals between successive records are not fixed: the average value between successive records is close to 1 min during 1984 and 1985 and close to 15 s during 1986–89. We define the trading time as a continuous time starting from the opening of the day until the closing, and then continuing with the opening of the next trading day. We checked that overnight price differences<sup>26</sup> do not affect the scaling properties of the stochastic process. From this data base, we select the complete set of non-overlapping records separated by a time interval  $\Delta t \pm \varepsilon \Delta t$  (where  $\varepsilon$  is the tolerance, always less than 0.035). We denote the value of the S&P 500 as  $y(t)$ , and the successive variations of the S&P index is  $Z(t) \equiv y(t) - y(t - \Delta t)$ .

To characterize quantitatively the experimentally observed process, we first determine the probability distribution  $P(Z)$  of index variations for different values of  $\Delta t$ . We select  $\Delta t$  values that are logarithmically equally spaced ranging from 1 to



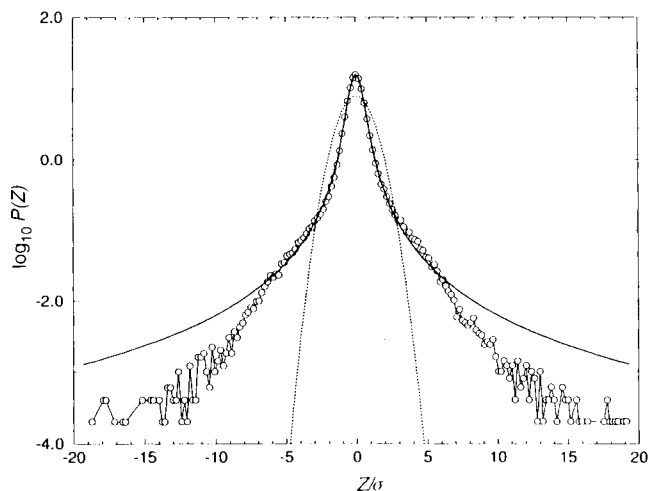


FIG. 2 Comparison of the  $\Delta t = 1$  min probability distribution with the symmetrical Lévy stable distribution of index  $\alpha = 1.40$  and scale factor  $\gamma = 0.00375$  (solid line). The scale factor  $\gamma$  is obtained from equation (2) by using the experimental values of  $\alpha$  and  $P(0)$ . The dotted line shows the gaussian distribution with standard deviation  $\sigma$  equal to the experimental value 0.0508. The variations of price are normalized to this value. Approximately exponential deviations from the Lévy stable profile are observed for  $|Z|/\sigma \gtrsim 6$ .

1,000 min. The number of data in each set decreases from the maximum value of 493,545 ( $\Delta t = 1$  min) to the minimum value of 562 ( $\Delta t = 1,000$  min).

Figure 1a is a semilogarithmic plot of  $P(Z)$  obtained for seven different values of  $\Delta t$ . As expected for a random process, the distributions are roughly symmetrical and are spreading when  $\Delta t$  increases. We also note that the distributions are leptokurtic, that is, they have wings larger than expected for a normal process. A determination of the parameters characterizing the distributions is difficult if one uses methods that mainly investigate the wings of distributions, especially because larger values of  $\Delta t$  imply a reduced number of data.

Therefore we use a different approach: we study the 'probability of return'  $P(Z=0)$  as a function of  $\Delta t$ . With this choice we investigate the point of each probability distribution that is least affected by the noise introduced by the finiteness of the experimental data set. In Fig. 1b, we show  $P(0)$  versus  $\Delta t$  in a log-log plot. The data are fitted well by a straight line of slope  $-0.712 \pm 0.025$ . We observe a non-normal scaling behaviour (slope  $\neq -0.5$ ) in an interval of trading time ranging from 1 to 1,000 min. This experimental finding agrees with the theoretical model of a Lévy walk or Lévy flight<sup>9-11</sup>. In fact, if the central region of the distribution is well described by a Lévy stable symmetrical distribution,

$$L_\alpha(Z, \Delta t) \equiv \frac{1}{\pi} \int_0^\infty \exp(-\gamma \Delta t q^\alpha) \cos(qZ) dq \quad (1)$$

of index  $\alpha$  and scale factor  $\gamma$  at  $\Delta t = 1$ , where  $\exp(-\gamma \Delta t |q|^\alpha)$  is the characteristic function of the symmetrical stable process, then the probability of return is given by

$$P(0) \equiv L_\alpha(0, \Delta t) = \frac{\Gamma(1/\alpha)}{\pi \alpha (\gamma \Delta t)^{1/\alpha}} \quad (2)$$

where  $\Gamma$  is the Gamma function. By using the value  $-0.712 \pm 0.025$  from the data of Fig. 1b we obtain the index  $\alpha = 1.40 \pm 0.05$ .

We also check if the scaling extends over the entire probability distribution as well as  $Z=0$ . To this end, we first note that Lévy stable symmetrical distributions rescale under the transformations

$$Z_s \equiv \frac{Z}{(\Delta t)^{1/\alpha}} \quad (3a)$$

and

$$L_\alpha(Z_s, 1) \equiv \frac{L_\alpha(Z, \Delta t)}{(\Delta t)^{1/\alpha}} \quad (3b)$$

Figure 1c shows the distributions of Fig. 1a plotted in scaled variables. All the data collapse on the  $\Delta t = 1$  min distribution when we use equations (3a) and (3b) with  $\alpha = 1.40$ . From Fig. 1c we conclude that a Lévy distribution describes well the

dynamics of the probability distribution  $P(Z)$  of the random process over time intervals spanning three orders of magnitude.

In Fig. 2, we compare the probability distribution observed for  $\Delta t = 1$  min with the Lévy stable distribution of index  $\alpha = 1.40$ . Note that the solid line is not simply a 'fit' to the data; rather, the appropriate scale factor  $\gamma = 0.00375$  is obtained by using the experimental value of  $P(0)$  and equation (2). A good agreement with the Lévy (non-gaussian) profile is observed for almost three orders of magnitude when  $|Z|/\sigma \lesssim 6$  and an approximately exponential fall-off from the stable distribution is observed for  $|Z|/\sigma \gtrsim 6$ ; here  $\sigma = 0.0508$  is the standard deviation. Our results show a clear deviation of the tails of the distribution from the Lévy profile.

The Lévy distribution has an infinite second moment (if  $\alpha < 2$ ). But our experimental finding of an exponential (or stretched exponential) fall-off implies that the second moment is finite, thereby resolving the question of how one could get around the problem of an infinite variance if the Lévy distribution is used to describe the price difference distribution<sup>24</sup>. This conclusion might at first sight seem to contradict our observation of Lévy scaling of the central part of the price difference distribution over fully three orders of magnitude. However, there is no contradiction, because (for example), a recent study<sup>27</sup> finds that Lévy scaling may hold over a long period of time for the dynamics of 'quasi-stable' stochastic processes having a finite variance.

By using the Berry-Esseen theorem<sup>28,29</sup>, we can estimate that the maximal time needed to observe convergence for the price differences to a gaussian process is of the order of 1 month. This estimate is obtained by using the experimental values of  $\langle |Z|^3 \rangle$  and  $\langle Z^2 \rangle$  observed in the high-frequency regime (for example at  $\Delta t = 1$  minute). For our data set, we measure  $\langle |Z|^3 \rangle = 0.605 \times 10^{-3}$  and  $\langle Z^2 \rangle = 0.00257$ , and we set an upper bound of the difference between the integral of the distribution and the corresponding asymptotic normal process of 0.1. Our estimate of roughly 1 month is in agreement with an independent empirical study of the distribution of daily, weekly and monthly returns, for which a progressive convergence to a gaussian process is found<sup>30</sup>.

We also investigate the scaling properties of  $P(0)$  within each year to determine if the scale index  $\alpha$  is highly fluctuating from year to year. The results of this analysis are shown in Fig. 3. We also show the line best fitting  $P(0)$  of the entire set of data. In different years, the graph of  $P(0)$  is always parallel to the overall behaviour (dotted line) in a log-log plot. This implies that the index  $\alpha$  is roughly constant over the years. The scale factor  $\gamma$  (related to the vertical position of data in Fig. 3) varies somewhat from year to year. It is higher (lower positions of the experimental points in the figure) for periods of higher 'volatility' (an economic term indicating higher values of the variance of the price variations). When the same analysis is performed monthly, similar conclusions are obtained:  $\alpha$  is roughly constant ( $\alpha =$

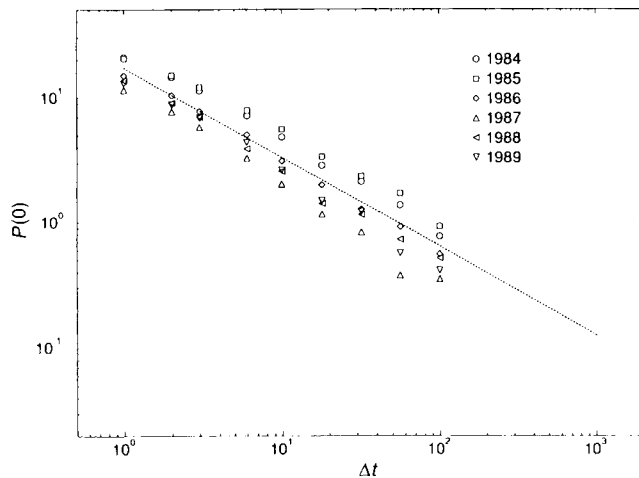


FIG. 3 Probability of return  $P(0)$  of the S&P 500 index variations as a function of the time sampling intervals observed in different years. The same scaling behaviour of the complete set of data (dotted line) is observed each year (data are parallel to the dotted line). The scale factor  $\gamma$  is slowly time-dependent, as the vertical positions of the probability of return change from year to year.

$1.38 \pm 0.14$ ) and  $\gamma$  fluctuates more than  $\alpha$ , having bursts of activity localized in specific months (such as April 1987, and October 1987 and immediately following months).

We compare our experimental results with the statistical properties of the heteroskedastic stochastic process GARCH(1, 1) (ref. 18). We simulate GARCH(1, 1) processes characterized by control parameters close to the values obtained in the time-series analysis of stock returns<sup>22</sup>. We find that the time evolution of the probability density functions (PDFs) of the GARCH(1, 1) process is quite different from that observed in the experimental data. In particular, we investigate the probability of return to the origin of the GARCH(1, 1) simulated process using the values of the control parameters selected to mimic the experimental  $\Delta t = 1$  min PDF, and find the data are fitted well by a straight line in a log-log plot, with slope  $-0.531 \pm 0.025$ . The scaling index is therefore  $1.88 \pm 0.09$ , a value close to 2 but 43% larger than the value of 1.4 observed in the experimental data. □

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## Dynamics of formation of symmetrical patterns by chemotactic bacteria

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MOTILE cells of *Escherichia coli* aggregate to form stable patterns of remarkable regularity when grown from a single point on certain substrates. Central to this self-organization is chemotaxis, the motion of bacteria along gradients of a chemical attractant that the cells themselves excrete<sup>1</sup>. Here we show how these complex patterns develop. The long-range spatial order arises from interactions between two multicellular aggregate structures: a 'swarm ring' that expands radially, and focal aggregates that have lower mobility. Patterning occurs through alternating domination by these two sources of excreted attractant (which we identify here as aspartate). The pattern geometries vary in a systematic way, depending on how long an aggregate remains active; this depends, in turn, on the initial concentration of substrate (here, succinate).

Under certain conditions, cells of chemotactic strains of *Escherichia coli* and *Salmonella typhimurium* excrete an attractant, aggregate in response to gradients of that attractant, and form patterns of varying cell density<sup>1–3</sup>. This process occurs only if cells are chemotactic towards aspartate, and can be suppressed by addition of aspartate or aspartate analogues. In *E. coli*, aggregates form in the wake of a travelling circular band, producing highly symmetrical patterns<sup>1</sup>. Their geometry depends on initial conditions; for example, the concentration of growth substrate. In *S. typhimurium*, aggregates arise from the centre of an unstructured bacterial lawn, producing patterns with lower symmetry<sup>2</sup>. The present work was undertaken to determine the mechanism by which *E. coli* forms patterns with long-range spatial order and why particular geometries arise at different initial concentrations of substrate. Succinate and fumarate both work well<sup>1</sup>; we used succinate.

Patterns appear within a certain range of succinate concentrations (Fig. 1). The concentrations of growth substrate required for each pattern vary from strain to strain, but for a given strain, the succession of patterns observed with increasing concentrations is fixed. At low concentrations of succinate, one sees a single compact travelling band (swarm ring) that moves slowly outwards (Fig. 1a). At higher concentrations, spots appear in concentric rings in radial rows (that is, on a pseudo-rectangular lattice), Fig. 1b, then in sets of intersecting spirals (that is, on a pseudo-hexagonal lattice), Fig. 1c, and finally on a pseudo-hexagonal lattice but with radial tails, Fig. 1d. As evident in Fig. 1d and documented below, the spots form in the wake of a band of cells moving outwards at the periphery of the pattern. An unstructured zone of low cell density always precedes the first element of a pattern. The radius of this zone decreases with increasing concentration of substrate. The radial periods and circumferential spacings differ from strain to strain, from about 1 to 6 mm. At even higher concentrations of substrate, one obtains more elaborate patterns, with radial streaks, indented rings, or petals (not shown). These patterns are not as reproducible, even in replicate plates, and some geometries appear only with certain strains.

To understand how changes in one initial condition, the concentration of succinate, can so dramatically alter the pattern-forming behaviour of the system, we studied effects of succinate concentration on bacterial growth and chemotaxis. The growth rate of *E. coli* in succinate saturates and is approximately constant over the concentration range 0.5–7 mM, in agreement with

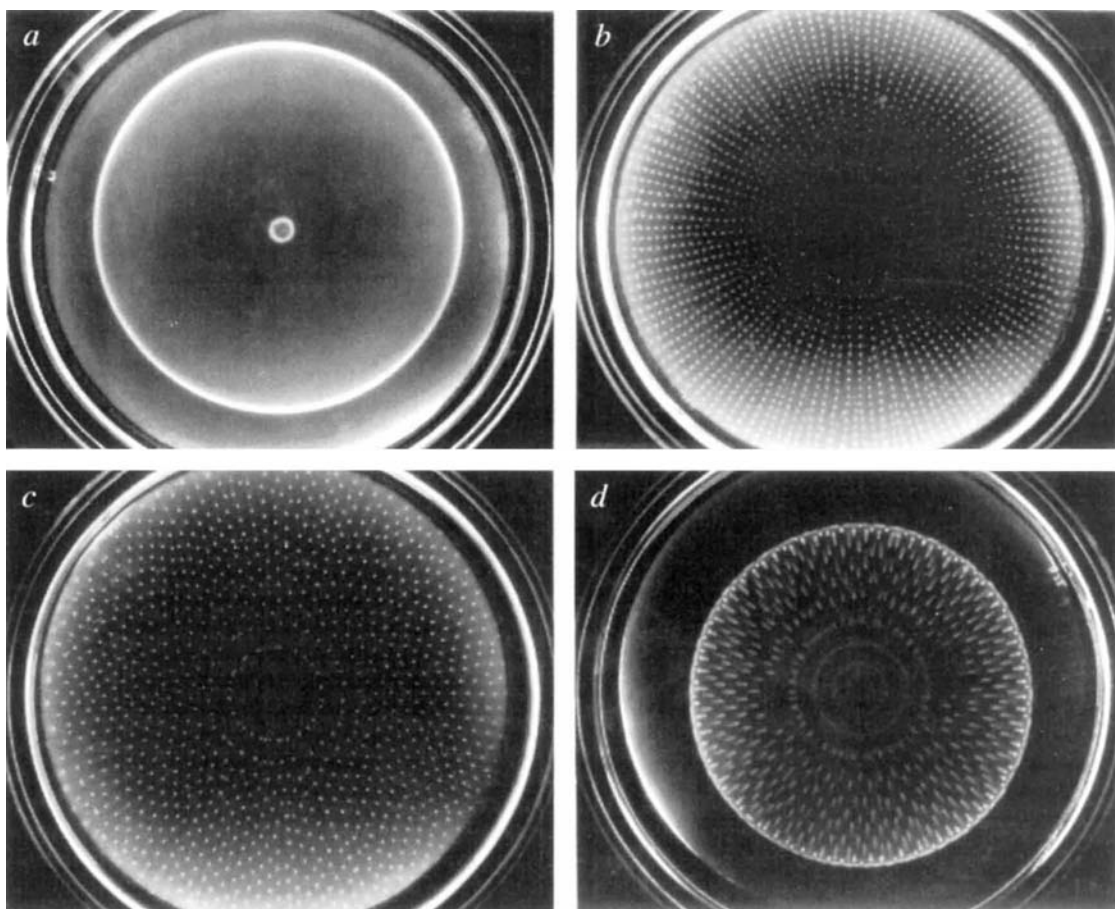


FIG. 1 Patterns generated by cells of *E. coli* chemotactic towards aspartate. With increasing concentrations of substrate (here, succinate) one obtains a swarm ring or a swarm ring followed by spots on a rectangular lattice, spots on a hexagonal lattice, or spots with tails on a hexagonal lattice (see ref. 1, Fig. 1b, a, d, respectively). a, A swarm ring generated by *E. coli* strain HCB317 (deleted for *tsr*, the gene that specifies the serine receptor<sup>14</sup>) grown on 1 mM succinate. An aspartate-blind mutant (not shown) generates, instead, an expanding disk of much lower, uniform cell density. b, Spots on a pseudo-rectangular lattice, generated by the same strain grown on 2 mM succinate. c, Spots on a pseudo-hexagonal lattice generated by the same strain grown on 3 mM succinate. d, Spots with tails on a pseudo-hexagonal lattice generated by cells of strain RP4368 (a serine-blind point mutant; from J. S. Parkinson) grown on 3 mM succinate. A similar pattern can be generated with

strain HCB317 (not shown).

**METHODS.** Growth was in plastic Petri plates (8.5 cm internal diameter) containing 10 ml of medium. Stock solutions of ingredients for M9 minimal medium<sup>15</sup> plus substrate and amino-acid supplements (L forms of threonine, leucine, histidine and methionine, 20  $\mu\text{g ml}^{-1}$  of each) were combined on each plate with 0.24% water agar (Difco Laboratories, Detroit). The vital dye tetrazolium red or tetrazolium violet (50  $\mu\text{g ml}^{-1}$ , Sigma) was added to improve image contrast. The plates were allowed to harden at room temperature on a level table for 2 h. Then they were inoculated at the centre with 5  $\mu\text{l}$  of fresh saturated culture, pregrown in M9-glycerol (33 mM) and incubated in a level humid chamber at 25 °C. They were photographed 38, 72, 72 and 48 h later, respectively, from above on Polaroid 667 film against a dark background, illuminated slantwise from below by two circular fluorescent lamps.

the classic Monod relation<sup>4</sup>. Under our conditions (see Fig. 1 legend) the doubling time is  $\sim 2$  h. On the other hand, the final cell density is roughly proportional to the initial concentration of succinate (Fig. 2a). The rate of excretion of the attractant aspartate also increases with the concentration of succinate (Fig. 2b). Finally, the rate of expansion of the swarm ring decreases with the concentration of succinate (Fig. 4c). With strain HCB317, the swarm ring forms after  $\sim 24$  h, then advances at a constant rate. If the concentration of succinate is greater than 1.3 mM, aggregates appear at  $\sim 32$  h.

The swarm ring of Fig. 1a is not the same as the chemotactic ring that appears when cells consume a metabolizable attractant<sup>5,6</sup>. In order for the ring of Fig. 1a to form, cells must excrete an attractant. This attractant is aspartate. Neither swarm rings nor patterns form in soft agar with an *aspA* mutant under any set of conditions. This mutant (*E. coli* strain C58 Asp 28, ref. 7) is motile and chemotactic but fails to excrete aspartate (E.O.B. and H.C.B., unpublished results). The defect is in aspartate (L-aspartate ammonia-lyase, EC 4.3.1.1), an enzyme that aminates fumarate.

It is likely that migration of the swarm ring requires an asymmetric aspartate profile, with a higher concentration of aspartate

at the leading edge of the ring than at its trailing edge. This would be expected if the cells consume a substantial fraction of the succinate, thus decreasing the rate of excretion of aspartate at the trailing edge (Fig. 2b). Because the cells grow at a constant rate, this takes longer at higher succinate concentrations, explaining why the rate of expansion of the swarm ring is smaller at higher concentrations (Fig. 2c). An alternative possibility for the generation of an asymmetric aspartate profile is that cells at the trailing edge of the swarm ring are starved of ammonia, and so metabolize aspartate at a greater rate<sup>2,8</sup>.

Time-lapse video recordings were made at higher magnification to reveal events that take place in the vicinity of the swarm ring (Fig. 3). Generation of the pseudo-rectangular lattice begins when the swarm ring breaks into discrete spots (Fig. 3a, 0 h). A spot forms at one point along the circumference, and then new spots appear in succession on each side, so that the ring fragments both clockwise and anticlockwise at the rate of about 5  $\text{cm h}^{-1}$  (not shown). The first set of aggregates is left behind, while the swarm ring continues on its way (2.5 h). Later, the aggregates disintegrate, and many of their cells stream forward to rejoin the swarm ring (3.8 h). A subset remains to mark the spot where a given aggregate first formed. Finally, new aggre-

gates form at the points where cells rejoined the swarm ring (5.9 h). Then the process recycles: compare 5.9 with 0 h, 7.2 with 2.5 h, and 8.5 with 3.8 h. A third set of aggregates appears at 9.5 h, and a fourth at 13.4 h. Thus, one obtains radial arrays of spots on concentric circles. At a large radius, when the circumfer-

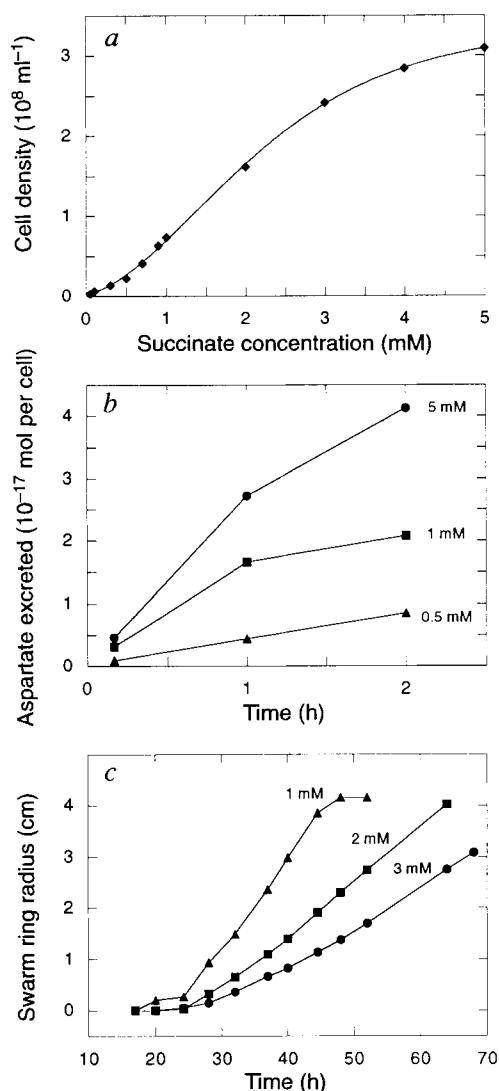


FIG. 2 Processes affected by substrate concentration. a, Final cell density; b, rate of excretion of aspartate; and c, radius of swarm rings, plotted as a function of time.

**METHODS.** All experiments were done with strain HCB317 (Fig. 1a) at 25 °C. a, Bacteria at low titre were inoculated in 10-ml aliquots of liquid medium (without agar) in Petri plates and incubated without shaking in the humid chamber. Final concentrations were determined from the absorbance at 600 nm, using the conversion factor for our spectrophotometer of  $8 \times 10^8$  cells per absorbance unit. Replicate plates gave essentially identical results. b, Cells were pregrown on M9-succinate (5 mM) to mid-exponential phase ( $1.4 \times 10^8$  cells per ml), then washed and incubated in 5 mM ammonium acetate (pH 7.0) supplemented with succinate at concentrations of 0.5, 1 and 5 mM at densities of 2.2, 2.0 and  $2.5 \times 10^8$  cells per ml, respectively. Aliquots were removed at different times, the cells were filtered out, the supernatant fraction was freeze-dried, and its aspartate content was determined in an amino-acid analyser (using pre-derivatization with phenylisothiocyanate, Applied Biosystems 420ABI). c, Agar plates with 1, 2 or 3 mM succinate (five at each concentration) were inoculated with 5  $\mu\text{l}$  of cells pregrown on M9-succinate (5 mM) to mid-exponential phase and diluted to  $1.2 \times 10^7$  cells per ml in M9 buffer. The diameter of the swarm ring was measured approximately every 3 h with a ruler. Mean radii are shown, limited to  $\sim 4$  cm by the size of the plate; the standard deviations were smaller than the symbols on the plots.

ential spacing exceeds a certain threshold, an intervening aggregate appears, and a new radial row is initiated (Fig. 1b).

With the pseudo-hexagonal lattice (Fig. 3b) there are two sets of aggregates forming and disintegrating at any one time, roughly half a cycle out of phase. Cells streaming toward the swarm ring from disintegrating aggregates in one row pass between aggregates that have just formed in the next row (0.6, 1.8 h). As before, new aggregates form where cells from earlier aggregates join the swarm ring. But now these aggregates tend to act independently: they move forward with the swarm ring, then stop and disintegrate. This independent behaviour is even more striking at higher substrate concentrations, when radial tails appear (Fig. 3c). Now, the nucleus of an aggregate moves as an intact structure, leaving cells in its wake that form a streak with diminishing width. The length of this streak (the radial tail) reflects the distance covered by the decaying aggregate.

When discrete aggregates first form, their cells are highly motile; after they disintegrate, the cells left behind are not. Therefore, stabilization of a pattern is achieved through loss of motility. Cells left behind in disintegrated aggregates were removed from plates and resuspended in motility medium<sup>9</sup>. Most were non-motile or failed to respond to aspartate. Some of these cells produced motile progeny after growth on hard agar, but others did not. Thus, the transition to the non-motile state is due to changes in both phenotype<sup>10</sup> and genotype.

How, then, are the patterns formed? The cells grow on succinate and excrete aspartate. A chemotactic response to aspartate, which is distributed asymmetrically as the cells grow, amplifies spatial inhomogeneity. A swarm ring forms at the periphery of the growing colony, and then the cells advance as a group away from the point of inoculation. Given time for cells to proliferate, the cell density within the swarm ring increases, and fluctuations in the local concentration of aspartate become large enough to trigger the formation of a discrete aggregate. Because the cell doubling time is constant whereas the rate of expansion of the swarm ring decreases with increasing substrate concentration (Fig. 2c), the first aggregate appears at a smaller radius at higher substrate concentrations (Fig. 1). Formation of this aggregate destabilizes the swarm ring. Other cells in the swarm ring move towards this aggregate, depleting regions to either side, generating a gradient of aspartate that is positive near the aggregate but negative farther away. Thus, more distant cells move away from the first aggregate, increasing the density farther away, where two new aggregates appear. This process continues in both directions around the ring until a complete set of aggregates has formed. Cells that are not recruited into this set remain in the swarm ring and continue on their way. Eventually, the density of the swarm ring increases to the point where new aggregates form. Naively, one would expect these aggregates to appear in between the earlier ones, as more cells remain in those regions after the first set has formed (for example, Fig. 3a, 7.2 h). Indeed, this is the way in which the hexagonal lattice is initiated (see below). However, at low concentrations of succinate, the original set of aggregates begins to run out of succinate and to excrete less aspartate. The gradient generated by the advancing swarm ring becomes dominant, and cells that remain chemotactic in the original set of aggregates stream forward to rejoin the swarm ring (for example, Fig. 3a, 8.5 h), leaving defective cells behind. This influx rapidly carries the local cell density over the aggregation threshold, and a new set of aggregates forms that are radially in-line with the first (for example, Fig. 3a, 9.5 h). The cells left behind are non-motile, and the pattern is frozen in.

At higher concentrations of succinate, cells remain in the aggregated state longer than they do at lower concentrations, mainly because it takes longer to use up the available succinate. Studies of time-lapse recordings made at the earliest stages of the development of hexagonal patterns show that the second set of aggregates tends to form in the swarm ring in between the first set; that is, the cell density there reaches threshold before the old aggregates begin to disintegrate (not shown). Once this



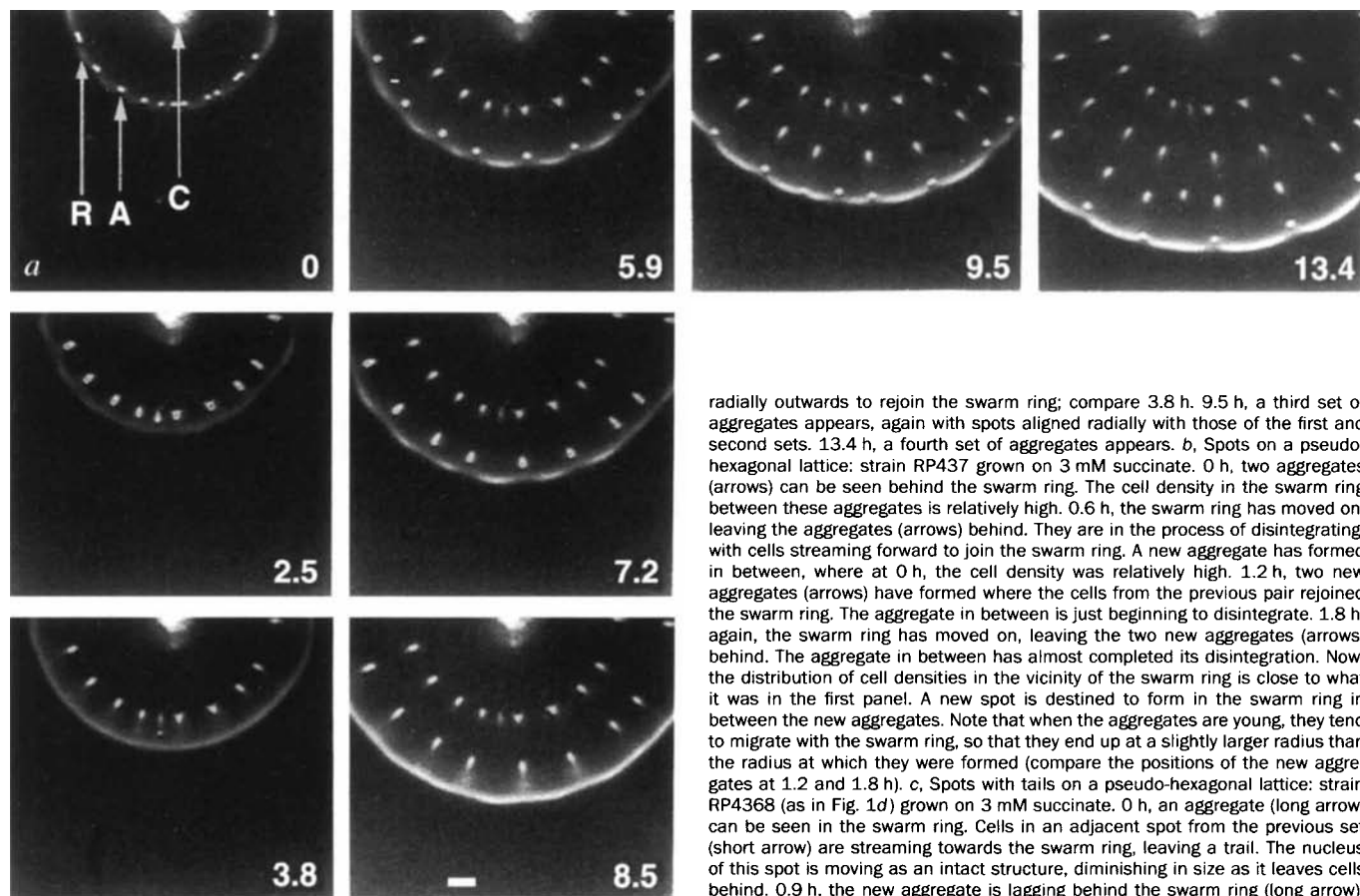
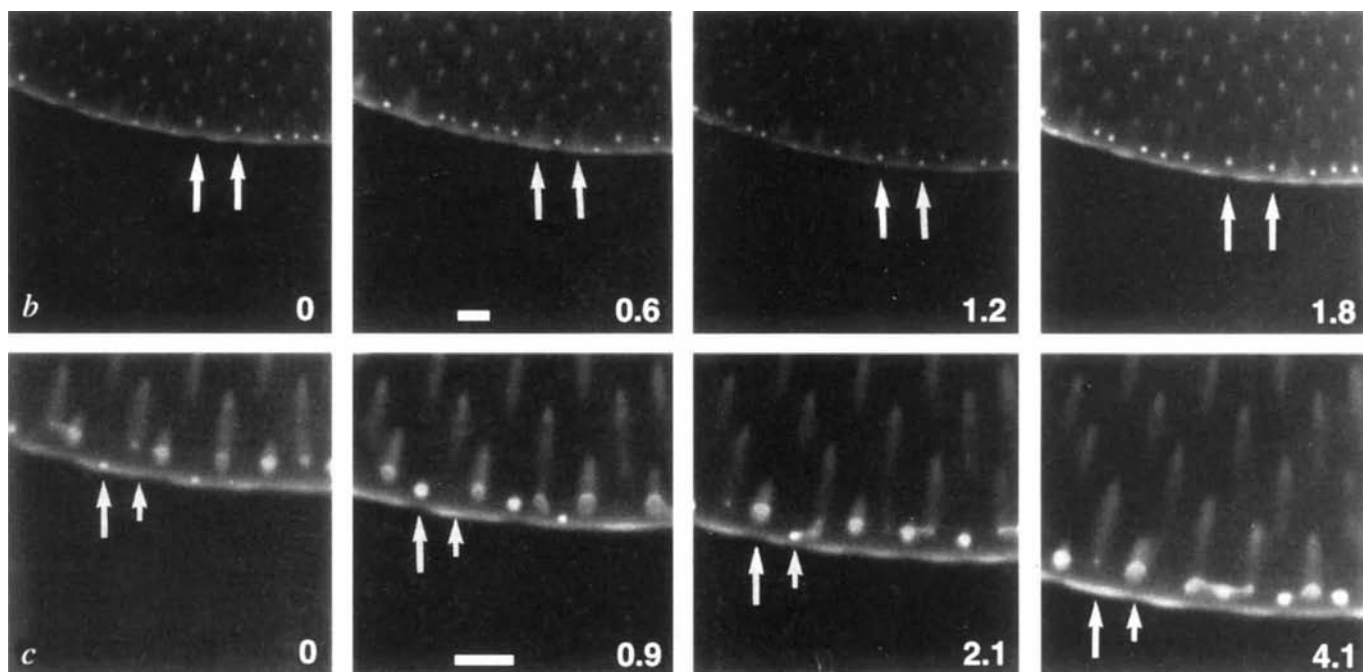


FIG. 3 Microscopic behaviour resulting in patterns of the kind shown in Fig. 1. The panels in each set of prints are labelled by elapsed time in h since the first panel. Scale bar, 2 mm. *a*, Spots on a pseudo-rectangular lattice: *E. coli* strain RP437 (wild-type for chemotaxis<sup>16</sup>) grown on 2 mM succinate. This pattern was recorded near the centre of the plate (C), where the spots are more widely dispersed. 0 h, an expanding swarm ring (R) breaking into aggregates (A). Note the stretches of swarm ring remaining between aggregates. 2.5 h, the swarm ring moves radially outwards, leaving the aggregates behind. The cell density in the swarm ring is higher between aggregates. 3.8 h, the aggregates disintegrate, and many of their cells stream radially outwards to rejoin the swarm ring. 5.9 h, a second set of aggregates appears in the swarm ring, with spots forming at the points of arrival of the cells from the first set. 7.2 h, once again, the swarm ring moves radially outwards, leaving the aggregates behind; compare with 2.5 h. 8.5 h, the second set of aggregates disintegrates, and many of their cells stream

radially outwards to rejoin the swarm ring; compare 3.8 h. 9.5 h, a third set of aggregates appears, again with spots aligned radially with those of the first and second sets. 13.4 h, a fourth set of aggregates appears. *b*, Spots on a pseudo-hexagonal lattice: strain RP437 grown on 3 mM succinate. 0 h, two aggregates (arrows) can be seen behind the swarm ring. The cell density in the swarm ring between these aggregates is relatively high. 0.6 h, the swarm ring has moved on, leaving the aggregates (arrows) behind. They are in the process of disintegrating, with cells streaming forward to join the swarm ring. A new aggregate has formed in between, where at 0 h, the cell density was relatively high. 1.2 h, two new aggregates (arrows) have formed where the cells from the previous pair rejoined the swarm ring. The aggregate in between is just beginning to disintegrate. 1.8 h, again, the swarm ring has moved on, leaving the two new aggregates (arrows) behind. The aggregate in between has almost completed its disintegration. Now, the distribution of cell densities in the vicinity of the swarm ring is close to what it was in the first panel. A new spot is destined to form in the swarm ring in between the new aggregates. Note that when the aggregates are young, they tend to migrate with the swarm ring, so that they end up at a slightly larger radius than the radius at which they were formed (compare the positions of the new aggregates at 1.2 and 1.8 h). *c*, Spots with tails on a pseudo-hexagonal lattice: strain RP4368 (as in Fig. 1*d*) grown on 3 mM succinate. 0 h, an aggregate (long arrow) can be seen in the swarm ring. Cells in an adjacent spot from the previous set (short arrow) are streaming towards the swarm ring, leaving a trail. The nucleus of this spot is moving as an intact structure, diminishing in size as it leaves cells behind. 0.9 h, the new aggregate is lagging behind the swarm ring (long arrow). The adjacent spot has finished its disintegration, and the swarm ring in front of it is more dense (short arrow). 2.1 h, the new aggregate is in the process of disintegration, its nucleus moving as a unit toward the swarm ring, leaving a trail (long arrow). Another aggregate has formed in the swarm ring at the point where cells from the adjacent spot joined it (short arrow). 4.1 h, the disintegration of the aggregate formed at 0 h is now nearly complete. The adjacent aggregate is beginning to disintegrate.

**METHODS.** As in Fig. 1, but recorded with a CCD camera (Hamamatsu XC-77 with C2400 control, with different extension tubes and a 50-mm lens) and a time-lapse video recorder (JVC BR-9000U, triggered manually by a homemade pulse generator). The fluorescent lamps were run continuously and cooled with a fan, and a sheet of heat-absorbing glass was placed below the Petri plate. The inside of the lid of the Petri plate was coated with a detergent (Triton X-100, Sigma) to prevent accumulation of droplets of condensed water. Selected frames were output on a video printer (Sony UP-870MD).



happens, a cycle is established in which adjacent aggregates in successive rows are born, mature and disintegrate in an alternating sequence (Fig. 3b). Finally, at even higher concentrations of succinate, local excretion of aspartate is sufficient to keep the centre of an aggregate intact, even when its cells are responding *en masse* to an external gradient. Less-responsive cells that fall behind generate tails which, like those of a comet, point outwards from the centre. Were this trend to continue, one would expect to find aggregates with long lifetimes that move about like multicellular organisms. *E. coli* does, in fact, form such structures (that we call 'slugs') and these orchestrate the development of the more elaborate patterns mentioned earlier (E.O.B. and H.C.B., manuscript in preparation).

In brief, excretion of aspartate by *E. coli* allows cells to form two well-defined multicellular structures, an advancing swarm ring and focal aggregates. Interactions between these structures determine the positions of emerging elements in the developing pattern. Models for pattern formation proposed thus far do not embody this mechanism. A continuous model by Bruno<sup>11</sup> assumes constant production of a stable attractant, and produces isolated spots without higher-order correlations. Continuous and discrete models by Ben-Jacob *et al.*<sup>12</sup> produce higher-order correlations, but with mechanisms requiring either autocatalytic production of attractant triggered by levels of toxic waste, or by addition of a second (repellent) chemotactic signal. We do not believe that either mechanism is required. In our view, variations in the rate of excretion of attractant determined by cell density and the availability of substrate will suffice. In any event, the chemotactic behaviour of the swarm ring and the focal aggregate should be modelled in detail; several questions need to be answered. How is the identity of the swarm ring maintained, and what determines its rate of migration? What dictates the separation of bacteria between swarm ring and aggregate? Under what conditions can an aggregate move as an intact structure, and what sets its life expectancy?

Formation of spatial patterns from a mass of initially identical cells is one of the central problems of developmental biology. Rapid progress has been made in understanding the genetic control of morphogenesis (see ref. 13 for a review). However, genetics does not always reveal the dynamics of interactions involved. As seen here, a simple system involving motile responses to a diffusible substance can have surprising consequences. Substrate consumption, cell proliferation, excretion of attractant, and chemotactic motility, when combined in a certain way, can generate complex spatial structures; a specialized morphogenetic program is not required. With bacteria, these processes can be modulated independently in a controlled manner, allowing accurate tests of quantitative models for pattern formation. □

## Terminal Proterozoic reorganization of biogeochemical cycles

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THE Proterozoic aeon (2,500–540 million years ago) saw episodic increases in atmospheric oxygen content<sup>1</sup>, the evolution of multicellular life<sup>2,3</sup> and, at its close, an enormous radiation of animal diversity<sup>3</sup>. These profound biological and environmental changes must have been linked, but the underlying mechanisms have been obscure. Here we show that hydrocarbons extracted from Proterozoic sediments in several locations worldwide are derived mainly from bacteria or other heterotrophs rather than from photosynthetic organisms. Biodegradation of algal products in sedimenting matter was therefore unusually complete, indicating that organic material was extensively reworked as it sank slowly through the water column. We propose that a significant proportion of this reworking will have been mediated by sulphate-reducing bacteria, forming sulphide. The production of sulphide and consumption of oxygen near the ocean surface will have inhibited transport of O<sub>2</sub> to the deep ocean. We find that preservation of algal-lipid skeletons improves at the beginning of the Cambrian, reflecting the increase in transport by rapidly sinking faecal pellets. We suggest that this rapid removal of organic matter will have increased oxygenation of surface waters, leading to a descent of the O<sub>2</sub>–sulphide interface to the sea floor and to marked changes in the marine environment, ultimately contributing to the Cambrian radiation.

Hydrocarbons extractable from sedimentary rocks of Proterozoic age are often enriched in <sup>13</sup>C relative to the associated insoluble organic debris<sup>4–6</sup>. When this same relationship, which reverses that found between extractable lipids and total biomass in living organisms, is observed in more recent strata, it usually indicates that the soluble compounds have migrated from some other source. That this is not the case for the Proterozoic sediments is confirmed by our present analyses of organic materials from well preserved Proterozoic units. As shown in Fig. 1a, *n*-heptadecane is enriched in <sup>13</sup>C relative to kerogen (for which  $\delta$  values vary due to secular trends that have been widely recognized as characteristic of Proterozoic strata<sup>7,8</sup>), but pristane, a probable product of the degradation of chlorophyll and thus related to primary inputs, is isotopically depleted. No known source of contamination or migrating fluids would provide this combination of enrichment and depletion.

The depletion of <sup>13</sup>C in lipids relative to total biomass is characteristic of all autotrophs and respiring heterotrophs. The reversed relationship shown in Fig. 1a cannot, therefore, indicate simply that Proterozoic organisms used different biosynthetic pathways and thus fractionated isotopes differently. Instead, an ecological factor, something related to the processing of organic material after biosynthesis but before burial, must be responsible. In Phanerozoic and modern systems, isotopic enrichment is associated with heterotrophic reworking<sup>9,10</sup>. It does not generally produce geolipids enriched in <sup>13</sup>C relative to coexisting kerogen, but could if effects were strongly accentuated and made appropriately selective at the molecular level.

Pathways of carbon flow during most of the Proterozoic differed from those in later times because organic matter pro-

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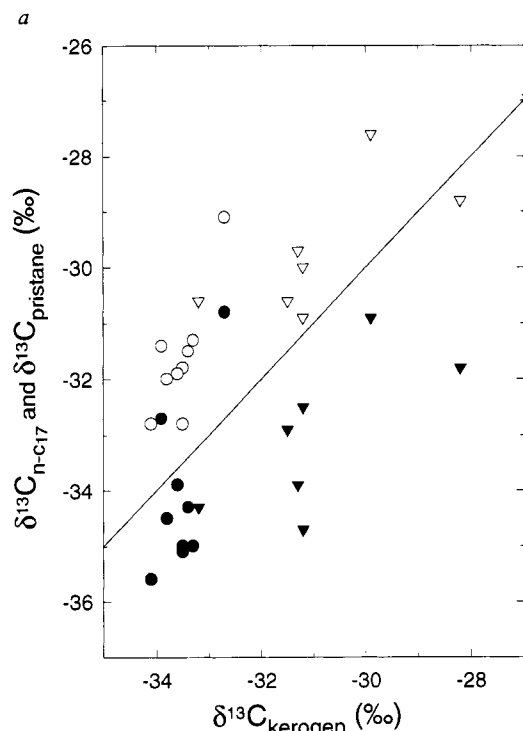
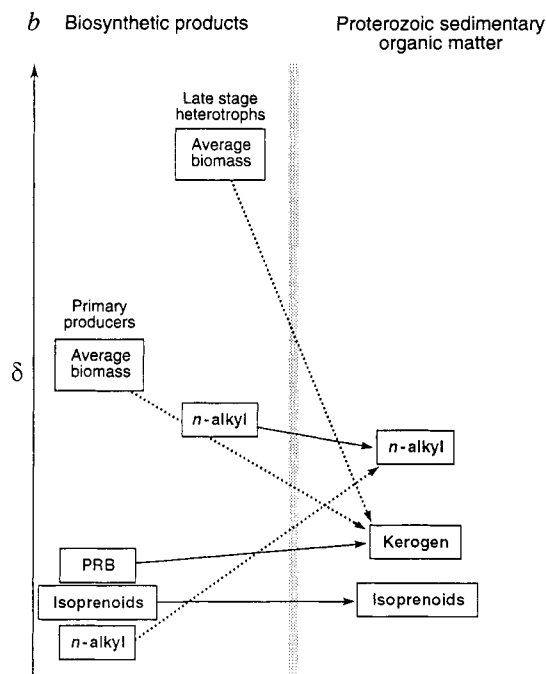


FIG. 1 a, Isotopic compositions of *n*-heptadecane (open symbols) and pristane (filled symbols) versus that of associated kerogen in samples from the Nonesuch Formation (circles, North American midcontinent rift, 1,055 Myr) and the Rodda beds (triangles, Officer basin, Australia, 590 Myr). The *n*-alkyl carbon skeleton is generally enriched in  $^{13}\text{C}$  compared to kerogen and plots above the 1:1 line whereas the isoprenoid hydrocarbon is depleted. The consistency of that pattern, together with the unprecedented combination of isotopic enrichment and depletion,

duced in surface waters sank much more slowly. It was not promptly repackaged as relatively dense, fast-sinking faecal matter—an important mechanism transporting organic matter to deep water in the modern ocean<sup>11</sup>—and would have descended only as individual particles or aggregates. In some areas, and at some times, ballasting by dust would be likely. Accordingly, degradation of organic matter must have been concentrated high in the water column. Substances escaping remineralization would have included refractory products of photosynthetic organisms and lipids from organisms near the end of the food chain. As shown in Fig. 1b, this combination explains the observed isotopic and structural relationships. The depletion of  $^{13}\text{C}$  in isoprenoid hydrocarbons relative to kerogen is similar to that observed in Phanerozoic samples and is consistent with derivation of the kerogen largely from refractory aliphatic biopolymers (algenans) produced by microalgae<sup>12,13</sup>. Heterotrophic bacteria produce much smaller amounts of refractory biopolymers and their contributions to kerogen are thus less significant. The enrichment of  $^{13}\text{C}$  in *n*-heptadecane indicates derivation mainly from the free lipids of late-stage heterotrophs, *n*-alkyl carbon skeletons of photosynthetic origin having been degraded. This unusual combination of primary and heterotrophic products is a logical result of conditions that were, as indicated by Fig. 2, characteristically Proterozoic. Moreover, its origin can be related simply and specifically to the absence of rapidly sinking particles.

The localization of heterotrophic activity high in the water column raises questions about the redox structure of the ocean. Independent evidence appears in the sedimentary record, which contains banded iron formations of late Proterozoic age, indicating that the deep ocean was at least intermittently anaerobic<sup>14</sup> even though oxygenic photosynthesis must have developed at least two billion years earlier and progressive oxidation of Earth's surface begun at that time<sup>1</sup>. Considering this problem,



provide evidence for the indigenous nature of the extractable components. b, Kerogen in Proterozoic sediments probably derives from resistant biopolymers of primary photosynthetic origin (PRB, *polymer resistant biologique*<sup>13</sup>) and condensation of some products from heterotrophic organisms. As a result, it is enriched in  $^{13}\text{C}$  relative to primary isoprenoids and depleted relative to *n*-alkyl carbon skeletons produced by heterotrophic organisms.

Holland<sup>15</sup> has called for 'the presence of other reductants [in addition to  $\text{Fe}^{2+}$  introduced by hydrothermal systems] to remove excess  $\text{O}_2$  from downwelling seawater.' A biogeochemical view based on the present evidence is summarized in Fig. 3a. Slow sinking of organic products would mean not only that carbon and nutrients were exported to the deep ocean less effectively but also that most photosynthetically produced oxygen was consumed where it was generated. Retention of nutrients in surface waters would enhance fertility<sup>8</sup>, but the feeble export of organic matter would create a compensatory demand for  $\text{O}_2$  not unlike that in modern surface waters made eutrophic by anthropogenic inputs of nutrients. Because some  $\text{O}_2$  would evade to the atmosphere (where at least part of it would be taken up by weathering reactions on the continents), supplies of oxidizable organic material would exceed those of  $\text{O}_2$  and a significant portion of the remineralization of organic matter would be mediated by sulphate-reducing bacteria. A 'biological redox buffer' would form, with sulphide and the slowly sinking organic matter serving as electron donors that preserved anaerobic conditions in the deep ocean.

Formation and preservation of carbonate minerals would have been affected. The simplified chemical reaction best representing remineralization of biomass by sulphate-reducing bacteria would be



As indicated, charge balance would be maintained without generation of  $\text{CO}_3^{2-}$ . Moreover, acidity would be generated in the form of  $\text{CO}_2$ . Although some of the  $\text{HS}^-$  would be reoxidized by aerobic chemoautotrophs, the production of sulphate would reduce alkalinity and not break the connection between remineralization of organic matter and dissolution of carbonate minerals. If anything, the carbonate lysocline would be shallower,

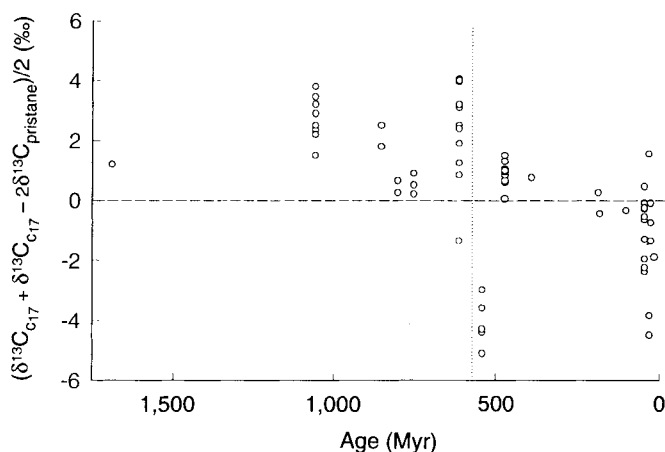


FIG. 2 Enrichment of  $^{13}\text{C}$  in linear alkanes (represented by *n*-heptadecane and *n*-octadecane) relative to pristane as a function of geological age. Unlike the lipid-versus-kerogen contrast emphasized in Fig. 1a, this parameter can reflect not only extensive heterotrophy but also the effect of non-algal sources of pristane. It is used in this long-time-base plot because data allowing isotopic comparisons of linear and isoprenoid lipids to kerogens are sparse. The graph includes all presently available analyses of sedimentary extracts and shows that the *n*-alkyl-versus-isoprenoid contrast shown in Fig. 1 is characteristic of Proterozoic units (to the left of the vertical broken line) and rare in Phanerozoic deposits. The points above the line at 450 and 40 Myr represent the Maquoketa Group and the Green River Shale respectively. Numerous other features of these extracts indicate stratification of the water column and the likely presence of non-algal sources of pristane<sup>34</sup> and thus perturbation of the isotopic signal. Stratigraphic units include the Barney Creek Formation, McArthur basin, Australia, 1,690 Myr (Proterozoic ages based on PPRG-P estimates<sup>33</sup>); Nonesuch Formation, USA, 1,055 Myr; Bitter Springs Formation, Amadeus basin, Australia, 800 Myr; Walcott Member, Chuar Group, USA, 850 Myr; Visingsö Group, Sweden, 775 Myr; Rodda beds, Officer basin, Australia, 590 Myr; Pertatataka Formation, Amadeus basin, Australia, 590 Myr; Observatory Hill Formation, Officer basin, Australia, Early Cambrian; Ouldburra Formation, Officer basin, Australia, Early Cambrian; Maquoketa Group, Illinois basin, USA, Ordovician<sup>34</sup>; Old Red Sandstone, Orkney, Scotland, Devonian; Paris Basin Shale, France, Toarcian<sup>35</sup>; Jurassic coal<sup>35</sup>; Green River Shale, USA, Eocene<sup>35</sup>; Green River Shale, USA, Eocene<sup>36</sup>; Mulhouse basin, Oligocene<sup>37</sup>; Baise Basin coal, Tertiary<sup>38</sup>.

possibly explaining the dominance of coastal carbonates in late Proterozoic strata<sup>16</sup>.

The processes outlined above also help to explain the unusual record of sulphur-isotope abundances in sediments of Proterozoic age. That record is problematical because, for more than  $10^9$  years during the Proterozoic, both sedimentary sulphides and evaporitic sulphates are enriched in  $^{34}\text{S}$  relative to the average prevailing during the Archean and Phanerozoic<sup>7,17</sup>. As this could occur only through violation of mass-balance requirements, the preserved record must be incomplete and biased<sup>18</sup>. Under the conditions described here, sulphides formed in open marine environments would be strongly depleted in  $^{34}\text{S}$ , as their formation within the water column would favour maximal isotopic fractionation<sup>19</sup>. Notably, new analyses of sulphides from rare deep-water facies in the southern Canadian Cordillera provide some of the first evidence of strongly depleted sulphides in sediments of Neoproterozoic age<sup>20</sup>. Incompleteness and bias would be introduced when sea-floor materials were preferentially removed from the record by subduction. As in later times, mass balance in the crust would be preserved by a combination of remobilization of sedimentary sulphur in island-arc volcanic systems<sup>7,21</sup> and mixing with seawater sulphate retained by oceanic basalts<sup>22</sup>.

An explanation is still required for the very strong enrichment of  $^{34}\text{S}$  in the sulphides which have survived, presumably as a

result of deposition in shelf environments. Where the  $\delta$  values approach but do not exceed those of coeval marine sulphate, it is required only that isotopic fractionation was minimized. This would be favoured in shallow waters because larger amounts of organic matter would be incorporated in sediments. There, particularly in the absence of bioturbation, sulphate-reducing bacteria would be cut off from supplies of sulphate beyond those present in pore waters and, as consumption of the sulphate proceeded, the  $\delta$  value of sedimentary sulphide would approach that of seawater sulphate (Fig. 3). The phenomenon would be accentuated by rapid burial and decreasing water depths and precisely these effects have been independently inferred in recent studies of Neoproterozoic sulphides<sup>20</sup>. For the rare instances in which sedimentary sulphides are isotopically enriched relative to seawater sulphate, additional processes can be suggested. First, drawdown of sulphate in the water column would create a sulphate-minimum zone (SMZ) analogous to the modern oxygen-minimum zone<sup>23</sup>. In extreme cases, the residual sulphate in the SMZ might have been significantly enriched in  $^{34}\text{S}$ . Where it impinged on coastal sediments, such an SMZ would have supplied pre-enriched sulphate to sedimentary communities. Second, colonization of the sediment-water interface by sulphate-reducing bacteria would create a situation in which the first-formed, isotopically depleted sulphide would be preferentially lost by diffusion into the water column. Sulphate available for diffusion into the pore waters, where precipitation and preservation of sulphide minerals would have been more efficient, would have been enriched in  $^{34}\text{S}$ .

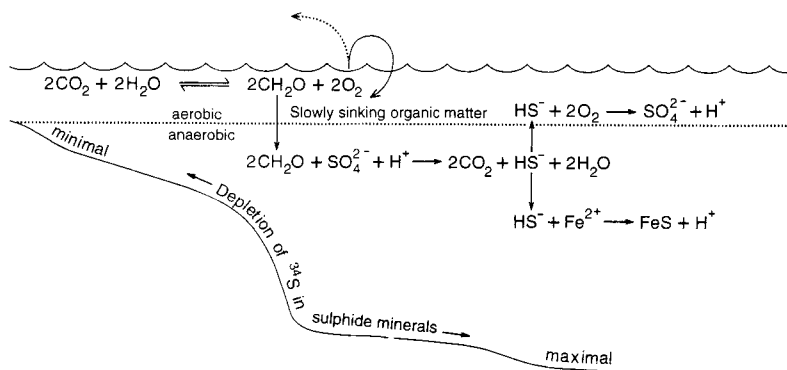
Additional geochemical signals can be correlated with the proposed environmental changes. For example, the percentage of carbon buried as organic matter rather than carbonate minerals decreased at the Proterozoic/Cambrian transition. This occurred even though rates of erosion, identified as the cause of enhanced burial of organic carbon during the Neoproterozoic, remained high<sup>24</sup>. The marked decrease in preservation of organic matter can now be attributed to oxygenation of the deep sea and the development of benthic metazoans which not only consumed organic carbon directly but also improved supplies of sulphate to bacteria through bioturbation and irrigation of sediments, thus enhancing anaerobic degradation. A second example concerns the global episode of phosphogenesis in the early Cambrian. Before faecal pellets were important, organic matter reaching the sediment-water interface was rarely rich in phosphorus<sup>8</sup> and sediments and bottom waters were commonly anaerobic. Subsequently, phosphorus was delivered to the sediment surface and aerobic conditions prevailed there, favouring retention of phosphate on sedimentary minerals<sup>25</sup>. The first phosphorites in this episode predate the Cambrian radiation<sup>26,27</sup>, indicating that oxygenation of the sea floor was gradual and that the most dramatic biological developments did not occur until the global ocean was ventilated.

Palaeontological and geochemical records reveal parallel developments at the end of the Proterozoic<sup>28</sup>: (1) carbon- and strontium-isotope abundances<sup>24</sup> indicate particularly significant burial of organic carbon and, thus, oxidation of the surface environment<sup>1,29</sup>; (2) ichnofossils representative of burrowing organisms<sup>30</sup> appear in shallow-water environments, indicating the emergence of triploblastic, coelomic metazoans; and (3) metazoan body fossils appear<sup>3</sup>. The development of metazoans was probably linked to the rise of  $\text{O}_2$  levels in surface environments<sup>31,32</sup>, but the present results suggest strongly that the explosive radiation of animal life at the beginning of the Cambrian period was dependent not on that evolutionary step but instead on a reorganization of biogeochemical cycles. The oldest rock units in which *n*-alkyl carbon skeletons are consistently depleted in  $^{13}\text{C}$  relative to the coexisting pristane and kerogen are the Early Cambrian Ouldburra Formation and Observatory Hill beds of South Australia (Fig. 2). As in modern settings, this improved preservation of algal products probably indicates that primary materials were then being transported

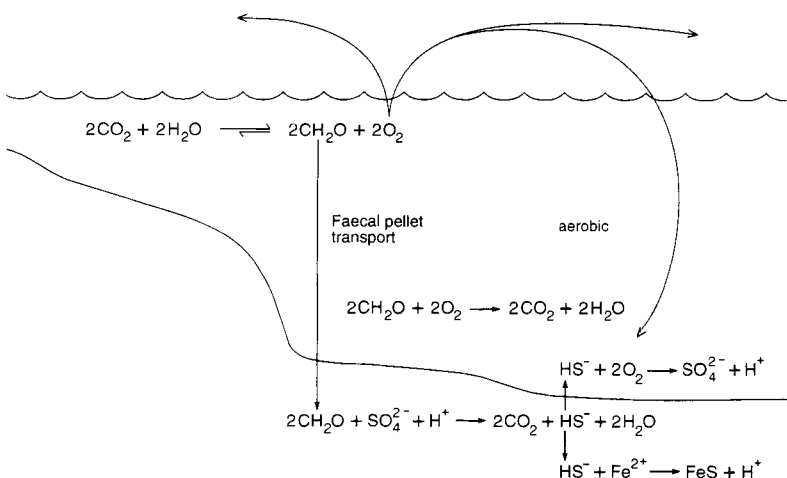


FIG. 3 Schematic representation of the 'reorganization of biogeochemical cycles.' a, Flows of organic carbon and  $O_2$  in Proterozoic oceans. Because of the slow sinking of organic matter, demands by heterotrophic organisms for  $O_2$  and other electron acceptors in surface waters were high. To whatever extent  $O_2$  escaped by evasion to the atmosphere, production of sulphide by planktonic sulphate-reducing bacteria is likely to have been important. Together with the slowly sinking organic matter itself, the sulphide will have retarded transport of  $O_2$  to deeper waters. b, Flows of organic carbon and  $O_2$  in Phanerozoic oceans. The rapid removal of organic carbon from surface waters reduces the demand for  $O_2$  and stimulates its transport throughout the environment. The delivery of appreciable quantities of organic carbon to the sea floor shifts terminal metabolism, and the associated production of sulphide, to that locale, moving the biological redox buffer from surface waters to the sediments.

### a Proterozoic ( $O_2$ largely retained in surface waters)



### b Phanerozoic (Greater release of $O_2$ from surface waters)



rapidly from surface waters. As illustrated in Fig. 3b, this export of organic matter decreased the consumption of  $O_2$  in surface waters and destroyed the redox buffer. Concentrations of  $O_2$  at the sea surface increased and much larger portions became available for evasion to the atmosphere and, given the collapse of the redox buffer, transport to the deep sea. The final stages of remineralization and the associated production of sulphide shifted from the upper water column to the sea floor and sediments. Ultimately, the effects of this reorganization of biogeochemical cycles were profound: the deep ocean was ventilated, the sea floor was opened to colonization by animals, and the metazoan radiation amplified. □

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# Isolation of a hyperthermophilic archaeum predicted by *in situ* RNA analysis

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A variety of hyperthermophilic bacteria and archaea<sup>1</sup> have been isolated from high-temperature environments by plating and serial dilutions<sup>2</sup>. However, these techniques allow only the small percentage of organisms able to form colonies, or those that are predominant within environmental samples, to be obtained in pure culture. Recently, *in situ* 16S ribosomal RNA analyses of samples from the Obsidian hot pool at Yellowstone National Park, Wyoming, revealed a variety of archaeal sequences, which were all different from those of previously isolated species<sup>3,4</sup>. This suggests substantial diversity of archaea with so far unknown morphological, physiological and biochemical features, which may play an important part within high-temperature ecosystems. Here we describe a procedure to obtain pure cultures of unknown organisms harbouring specific 16S rRNA sequences identified previously within the environment. It combines visual recognition of single cells by phylogenetic staining<sup>5-9</sup> and cloning by 'optical tweezers'<sup>10,11</sup>. Our result validates polymerase chain reaction data on the existence of large archaeal communities.

To isolate hyperthermophilic archaea, aerobic and anaerobic water and sediment samples were taken from the 'Obsidian Pool' located in the Mud Volcano area of Yellowstone National Park, Wyoming<sup>3</sup>. The samples were collected at different sites with a pH of 6.7 and temperatures between 73 and 93 °C. In the laboratory, 75 successful enrichment cultures were obtained (see legend to Fig. 1), which contained mixtures of coccoid-, rod- and plate-shaped cells differing in length and diameter. Whole-cell hybridization of the enrichment cultures with fluorescently labelled oligonucleotide probes targeted to an archaea-specific region within the 16S rRNA<sup>7</sup> revealed the presence of archaeal cells within about 55% of the enrichment cultures (Fig. 1).

Within the archaea-containing enrichments, we wanted to search specifically for archaea harbouring 16S rRNA sequences that were so far known only from *in situ* phylogenetic analyses. In a first attempt, we chose sequence pSL91 (ref. 4) and designed a fluorescently labelled oligonucleotide probe with specificity for this sequence. Use of this probe in whole-cell hybridization revealed one enrichment culture with a positive hybridization

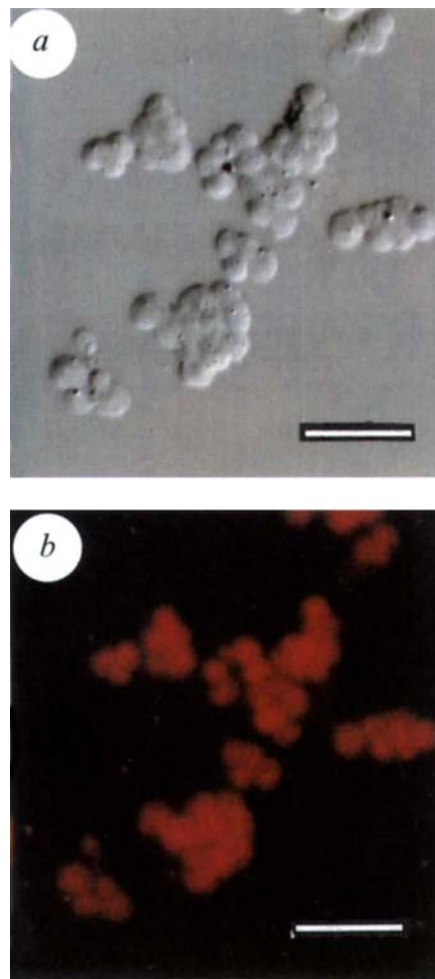
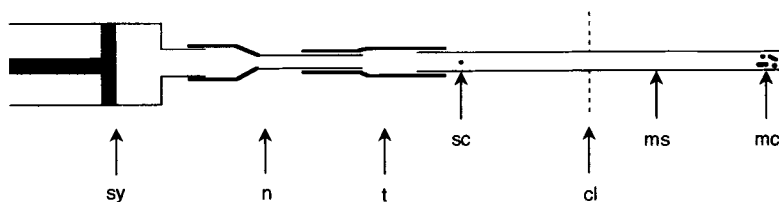


FIG. 1 Micrographs of isolate M11TL, taken on a confocal laser scanning microscope (LSM310, Zeiss, Oberkochen, Germany). Scale bar: 10  $\mu$ m. a, Nomarski interference contrast of grape-like aggregates of cocci. b, Whole-cell hybridization with a fluorescently labelled probe specific for clone pSL91 (ref. 4) performed as described<sup>6,9</sup>. The probe had the sequence 5'-TATGGGGGATTAGCACCA-3' and corresponded to positions 164 to 181 in the 16S rRNA (*Escherichia coli* numbering<sup>12</sup>). Hyperthermophilic organisms were enriched at 73, 83 and 93 °C, at pH 7, within anaerobic cultivation media containing possible energy sources<sup>13-18</sup>. Ionic strength had been adjusted to that of the original samples. Growth was observed by microscopic inspection. Portions (3 ml) of the cultures were processed for whole-cell hybridization<sup>6,9</sup>.

signal. It had been grown anaerobically at 83 °C with cell extracts as a heterogeneous energy source. Very rarely, based on their positive hybridization signal, grape-like aggregates of coccoid cells became visible, which had never been seen before

FIG. 2 Cell separation unit (schematic drawing). A rectangular microslide (ms) as observation chamber (inside dimension 0.1  $\times$  1 mm<sup>2</sup>; length 10 cm) was connected with a tube (t) to the needle (n) of a 1-ml syringe (sy) filled with sterile medium. From the open end, ~1  $\mu$ l of a mixed culture (mc) was soaked into the microslide. The microslide was fixed to the microscope stage of an inverted microscope (Axiovert IM35, Zeiss, Oberkochen, Germany) equipped with an oil immersion objective (100/1.3) and a Nd-YAG laser (wavelength 1064 nm; maximum power 1 W; ADLAS, Lübeck, Germany). From the cell suspension, inspected under 1000-fold magnification, a single cell (sc) was optically trapped in the laser beam and separated from the other cells by moving the microscope stage. Afterwards, the capillary was cut into two pieces (cutting line, cl) with the single cell on one side. The cell was flushed into fresh anaerobic medium and incubated at 83 °C.



in terrestrial hot springs. Within this enrichment culture, they were overdominated by at least four orders of magnitude by filamentous *Thermofilum*- and *Thermoproteus*-like cells. Isolation by conventional methods like serial dilutions and plating was therefore impossible. To clone the novel organism we employed a new micromanipulation technique consisting of a modified computer-controlled inverse microscope equipped with a strongly focused infrared laser ('optical tweezers')<sup>10,11</sup>. Cells were separated directly under visual control from the enrichment mixture within a square capillary 10 cm long connected to a sterile syringe (see Fig. 2). The single cells were then injected into sterile anaerobic culture medium. Within one week of incubation at 83 °C, about 30% of the cloned cells gave rise to pure cultures of grape-like aggregates. To confirm the phylogenetic position of the isolate (designated M11TL), its 16S rDNA was sequenced and shown to be indistinguishable from sequence pSL91 from the natural environment<sup>4</sup>. In this way, we have shown for the first time the identity of a sequence determined without cultivation from the native habitat with that of a pure culture.

Our results demonstrate that combining 16S rRNA analyses *in situ*, specific whole-cell hybridization within enrichments, and cloning under the laser microscope is a powerful approach for gaining a deeper understanding of microbial ecosystems and their participants. □

## Mesoderm induction in *Xenopus* caused by activation of MAP kinase

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MESODERM induction is a critical early step in vertebrate development, involving changes in gene expression and morphogenesis<sup>1</sup>. In *Xenopus*, normal mesoderm formation depends on signalling through the fibroblast growth factor (FGF) tyrosine kinase receptor<sup>2-4</sup>. One important signalling pathway from receptor tyrosine kinases involves p21<sup>ras</sup> (ref. 5). Ras associates with the serine kinase c-Raf-1 in a GTP-dependent manner, and this complex phosphorylates and activates MAPK/ERK kinase (MEK), a protein kinase with dual specificity. MEK then activates p42<sup>mapk</sup> and (at least in mammals) p44<sup>mapk</sup>, members of the mitogen-activated protein (MAP) kinase family<sup>6</sup>. FGF activates MAP kinase during mesoderm induction<sup>7-9</sup>, and the use of dominant-negative constructs suggests that mesoderm induction by FGF requires both Ras and Raf<sup>10,11</sup>. However, these experiments do not reveal whether Ras and Raf do act through MAP kinase to induce mesoderm or whether another pathway, such as the phosphatidylinositol 3-kinase cascade<sup>12</sup>, is involved. Here we show that expression of active forms of MEK or of MAP kinase induces ventral mesoderm of the kind elicited by FGF. Overexpression of a *Xenopus* MAP kinase phosphatase blocks mesoderm induction by FGF, and causes characteristic defects in mesoderm formation in intact embryos, whereas inhibition of the PI3 kinase and p70 S6 kinase pathways has no effect on mesoderm induction by FGF. FGF induces different types of mesoderm in a dose-dependent manner<sup>13</sup>; strikingly, this is mimicked by expressing different levels of

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activated MEK. Together, these experiments demonstrate that activation of MAP kinases is necessary and sufficient for mesoderm formation.

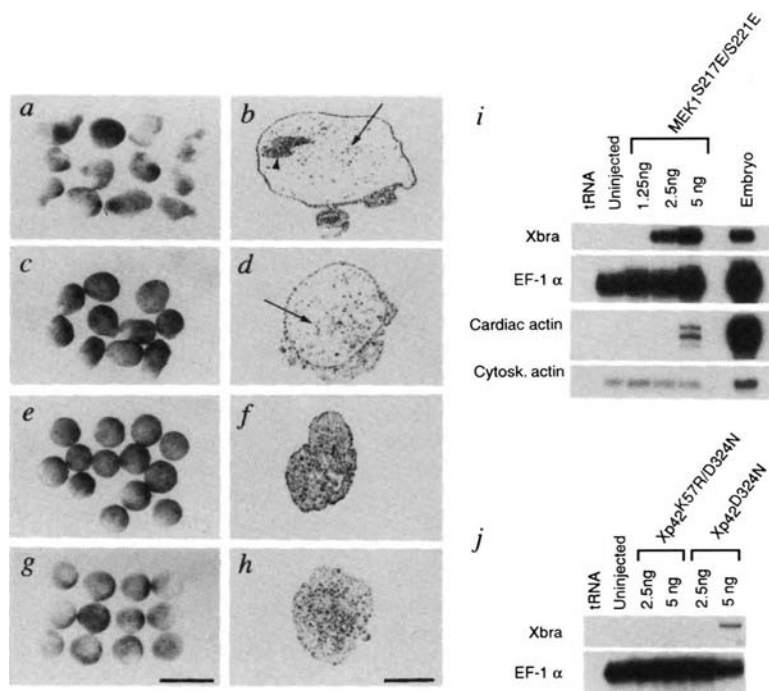
The mesoderm-inducing activities of MEK1 and *Xenopus* p42<sup>mapk</sup> (Xp42) were tested by injecting RNA encoding gain-of-function mutant forms of both proteins into fertilized eggs before dissecting animal caps at the blastula stage. We used a constitutively active form of MEK1 (MEK1<sup>S217E/S221E</sup>) in which serines 217 and 221 are replaced by glutamic acid<sup>14,15</sup>, and a modified form of Xp42 (Xp42<sup>D324N</sup>) in which the aspartate residue at position 324, located at the C-terminal end of kinase domain XI, is replaced by an asparagine residue. The equivalent mutation in the *Drosophila* MAP kinase DmERKA leads to the gain-of-function mutation sevenmaker, which activates multiple signalling pathways controlled by receptor tyrosine kinases such as sevenless and torso<sup>16</sup>. As a control, Xp42<sup>D324N</sup> was further modified by replacing lysine 57, an essential residue for kinase activity, with an arginine, to create Xp42<sup>K57R/D324N</sup>.

Animal caps derived from embryos injected with MEK1<sup>S217E/S221E</sup> RNA or with Xp42<sup>D324N</sup> RNA underwent elongation movements similar to those caused by FGF, whereas those derived from embryos injected with Xp42<sup>K57R/D324N</sup> RNA remained spherical and were indistinguishable from uninjected control caps (Fig. 1a, c, e, g). By the tadpole stage, animal caps expressing MEK1<sup>S217E/S221E</sup> or Xp42<sup>D324N</sup> had formed large vesicles. Histological analysis showed that these explants contained ventral mesodermal tissues including mesenchyme. Animal caps derived from embryos injected with MEK1<sup>S217E/S221E</sup> also formed muscle (Fig. 1b, d). The MEK1<sup>S217E/S221E</sup> construct therefore exerted a more pronounced effect on mesoderm induction than Xp42<sup>D324N</sup>, consistent with observations in tissue culture demonstrating that MEK1<sup>S217E/S221E</sup> has a stronger gain-of-function than sevenmaker<sup>17</sup>. Notochord formation was not observed in response to either construct. Uninjected caps and caps derived from Xp42<sup>K57R/D324N</sup>-injected embryos did not contain mesodermal tissue (Fig. 1f, h).

Mesoderm formation was also assessed by analysis of *Xbra* expression and cardiac actin expression. *Xbra* is expressed throughout the mesoderm of the early gastrula<sup>18</sup> and cardiac actin is expressed in somitic muscle<sup>19</sup>. Because higher doses of FGF are required to activate cardiac actin expression than are required to induce *Xbra*<sup>13</sup>, we investigated whether the injection

FIG. 1 Induction of mesoderm in blastula animal caps by active forms of MEK1 and of p42<sup>mapk</sup>. Morphological (a, c, e, g), histological (b, d, f, h) and molecular (i, j) analyses of mesoderm formation in animal caps derived from embryos injected with 5 ng MEK1<sup>S217E/S221E</sup> RNA (a, b), 5 ng Xp42<sup>D324N</sup> RNA (c, d), 5 ng Xp42<sup>K57R/D324N</sup> RNA (e, f) or from uninjected embryos (g, h). Animal caps derived from embryos injected with MEK1<sup>S217E/S221E</sup> or with Xp42<sup>D324N</sup> RNA undergo elongation. MEK1 causes formation of mesenchyme (arrow) and muscle (arrowhead) whereas Xp42<sup>D324N</sup> induces mesenchyme (arrow). Elongation and mesoderm formation are not observed in animal caps derived from embryos injected with Xp42<sup>K57R/D324N</sup> RNA or from uninjected embryos. i, Injection of MEK1<sup>S217E/S221E</sup> RNA causes activation of *Xbra* expression and of cardiac actin. j, Injection of Xp42<sup>D324N</sup> RNA induces expression of *Xbra*; tRNA, transfer RNA.

**METHODS.** *Xenopus* embryos were obtained by standard techniques and staged as described<sup>23</sup>. Complementary DNA encoding MEK1<sup>S217E/S221E</sup> was subcloned into pSP64T by blunt-ended ligation of a partial *EcoRI* digest of a pGEX3-MAPKK1<sup>S217E/S221E</sup> construct<sup>15</sup>. pSP64-Xp42 and pSP64-Xp42<sup>K57R</sup> were provided by J. A. Cooper. Site-directed mutagenesis was performed by overlap extension with the polymerase chain reaction<sup>24</sup>. The oligonucleotide 5'-GACCCAAGTAATGAGCCTGTAGCTGAGGC-3' was used to introduce a single G to A nucleotide change altering the Asp 324 codon to an Asn codon in Xp42 as well as in Xp42<sup>K57R</sup> (ref. 25). The flanking primers were: 5'-TGCAGG-ACCTCATGGAGACA-3' and 5'-TCTAGAGTCGTCGACCTGCAG-3'. The final PCR product was digested with *PstI* and *BglII* and introduced into pSP64-Xp42 and into pSP64-Xp42<sup>K57R</sup>, both of which were digested with *PstI* and *BglII*. Mutagenesis was confirmed by sequencing. Synthesis and microinjection of RNA were as described<sup>26</sup>. Animal caps were dissected at stage 8 with fine watchmaker's forceps and cultured in 75% NAM. They were frozen at stage 18 for RNase protection analysis with probes specific for *Xbra*, when EF-1 $\alpha$  was used as a loading control,



or cardiac actin, when cytoskeletal actin was used as loading control. Histological analysis was carried out after culture to the equivalent of stage 40. Analysis of morphogenetic movements was made at stage 16. RNA extraction and RNase protections with probes specific for *Xbra*, EF-1 $\alpha$  and cardiac actin were as described<sup>3</sup> and histological analysis was as described<sup>27</sup>.

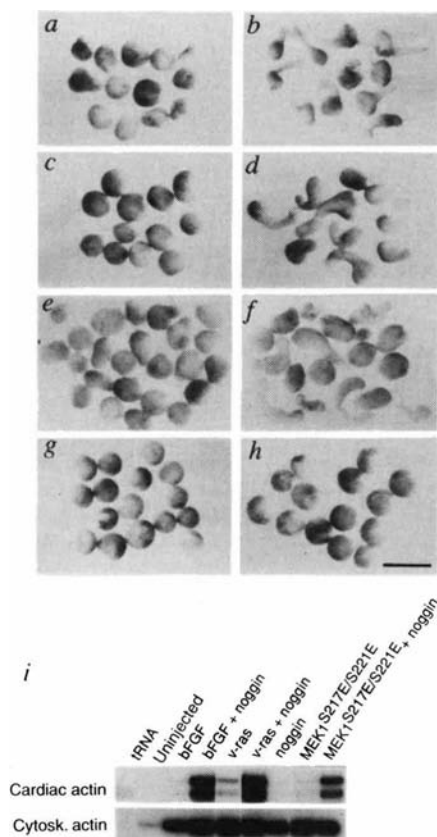


FIG. 2 Ventral mesoderm induced by components of the MAP kinase pathway can be dorsalized by *noggin*. Morphological (a-h) and molecular (i) analyses of the effects of *noggin* protein on mesoderm formation in animal caps incubated in 20 units bFGF per ml (a, b) or in caps derived from embryos injected with 100 pg p21<sup>v-Ha-ras</sup> RNA (c, d) or 5 ng MEK1<sup>S217E/S221E</sup> RNA (e, f), or in control caps. Animal caps in b, d, f and h were derived from embryos that had also been injected with 200 pg *noggin* RNA; *noggin* alone does not cause elongation, but it enhances morphogenetic movements induced by bFGF, v-ras and MEK1<sup>S217E/S221E</sup>. i, *noggin* alone does not induce expression of cardiac actin in *Xenopus* animal caps, but it acts synergistically with bFGF, v-ras and MEK1<sup>S217E/S221E</sup>.

**METHODS.** *Xenopus* embryos were injected with RNA encoding p21<sup>v-Ha-ras</sup> (ref. 11), MEK1<sup>S217E/S221E</sup> or *noggin*<sup>28</sup>, or combinations thereof, as described in the legend to Fig. 1. Animal caps were dissected at stage 8, treated where appropriate with bFGF, and cultured to stage 16 for assessment of elongation or stage 22 for RNase protection analysis. RNase protections with a probe specific for cardiac actin were done as described in the legend to Fig. 1.

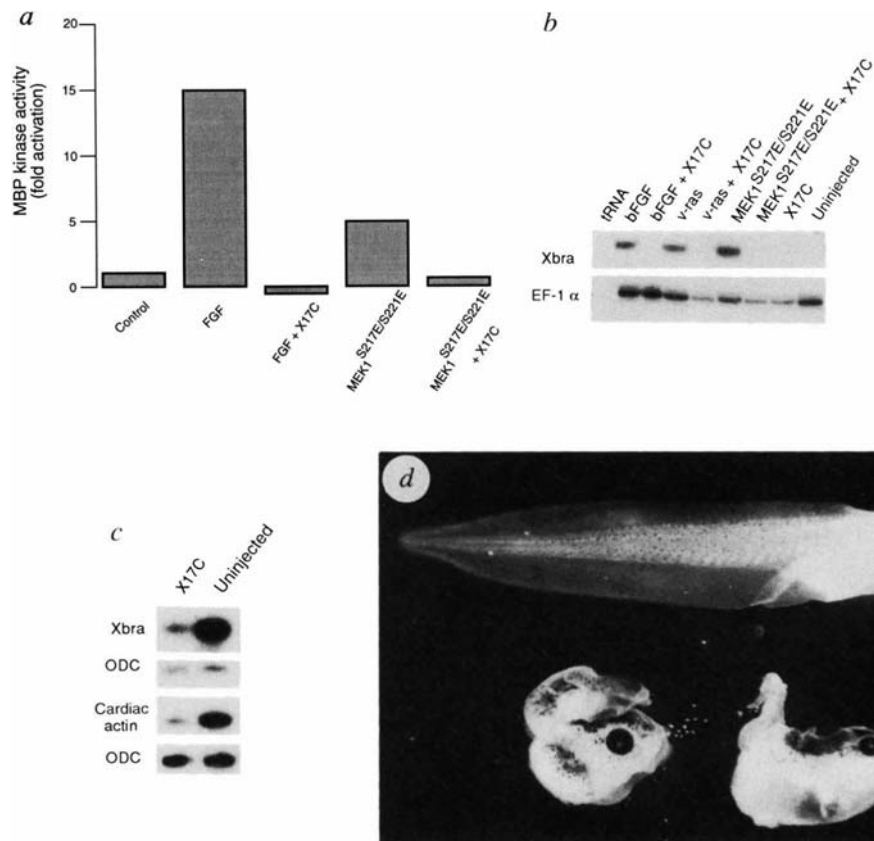


FIG. 3 A MAP-kinase-specific phosphatase inhibits mesoderm induction by FGF and interferes with posterior mesoderm formation in *Xenopus* embryos. **a**, MAP-kinase-specific phosphatase X17C inhibits MAP kinase activation due to FGF and to MEK1<sup>S217E/S221E</sup>. **b**, RNase protection analysis demonstrates that MAP-kinase-specific phosphatase X17C inhibits *Xbra* expression in response to FGF, v-ras and MEK1<sup>S217E/S221E</sup>. **c**, Intact embryos injected with RNA encoding MAP-kinase-specific phosphatase X17C were analysed by the reverse transcription-polymerase chain reaction (RT-PCR) technique for expression of *Xbra* at stage 11 or muscle-specific actin at stage 16. Expression of both mesodermal markers was inhibited by X17C. Ornithine decarboxylase (ODC) serves as a control for template level. **d**, Morphological effects of injecting X17C RNA into *Xenopus* embryos. The upper embryo is a control, and the two lower embryos represent the range of axial defects caused by injection of X17C RNA. There is a severe truncation of the posterior axis. Such truncations were never observed in embryos injected with water.

**METHODS.** X17C was cloned into the vector pSP64T, and RNA was transcribed essentially as described in the legend to Fig. 1. RNA (5–10 ng) was injected into each blastomere of the 2-cell embryo. In some experiments RNA encoding v-ras or MEK1<sup>S217E/S221E</sup> was also injected. Animal caps were dissected at stage 8 and cultured in the presence or absence of bFGF. MAP kinase assays were carried out by immunoprecipitation with antibody 122 (ref. 29), essentially as described<sup>29</sup>, except that samples were spotted onto P81 paper, washed with 75 mM orthophosphoric acid, dried, and analysed by Cerenkov counting. RNase protection analysis was done as described in the legend to Fig. 1. For RT-PCR, total RNA was isolated from whole embryos<sup>30</sup>. DNA was removed by treatment with DNase I. Random primed reverse transcription was done with 0.5 µg of input RNA in a reaction volume of 30 µl. Of this product,

of different amounts of MEK1<sup>S217E/S221E</sup> RNA would elicit differential gene expression in a similar fashion. RNase protection analysis showed that *Xbra* is strongly expressed in animal caps derived from embryos injected with 2.5 ng of MEK1<sup>S217E/S221E</sup> RNA, but injection of 5 ng RNA is required for expression of cardiac actin (Fig. 1i). Similarly, the injection of 5 ng of Xp42<sup>D324N</sup> RNA was sufficient to induce expression of *Xbra* in animal caps (Fig. 1j), but 10 ng RNA was required to induce cardiac actin (results not shown). Increasing levels of MEK or MAP kinase activity therefore elicit a pattern of thresholds similar to that observed in response to increasing concentrations of inducing factors. Animal caps derived from uninjected embryos, or from those expressing Xp42<sup>K57R/D324N</sup>, did not express *Xbra* or cardiac actin.

These results show that the expression of active forms of MEK1 or MAP kinase causes the formation of ventral mesoderm similar to that induced by FGF. One property of ventral mesoderm is that it can be dorsalized by the secreted protein noggin<sup>20</sup>. We find that the same is true for mesoderm induced by components of the MAP kinase pathway; *noggin* causes animal caps expressing v-ras or MEK1<sup>S217E/S221E</sup> to undergo greater elongation (Fig. 2a–h) and to express significantly increased levels of cardiac actin (Fig. 2i), both of which are characteristics of dorsal mesoderm<sup>20,21</sup>.



1 µl was taken for PCR amplification under conditions that ensured a direct correlation between RNA template abundance and amount of PCR product<sup>31</sup>. PCR products were radiolabelled by inclusion of trace [ $\alpha$ -<sup>32</sup>P]dGTP in the reaction, and they were analysed by autoradiography of polyacrylamide gels. The primer pairs were as follows (all written 5' to 3'): *Xbra* (ref. 18), GGATCGTTATCACCTCTG, GTGTAGTCTGTAGCAGCA; cardiac actin<sup>32</sup>, TCCCTGTACGCTTCTGGTCTGA, TCTCAAAGTCCAAAGCCACATA; ODC<sup>33</sup>, GGAGCTGCAAGTT-GGAGA, TCAGTTGCCAGTGTGGTC. The PCR cycle was: 94 °C for 30 s; 55 °C (or 62 °C for actin) for 1 min; 72 °C for 1 min. For actin and ODC, 19 cycles were performed; for *Xbra* 25 cycles were performed.

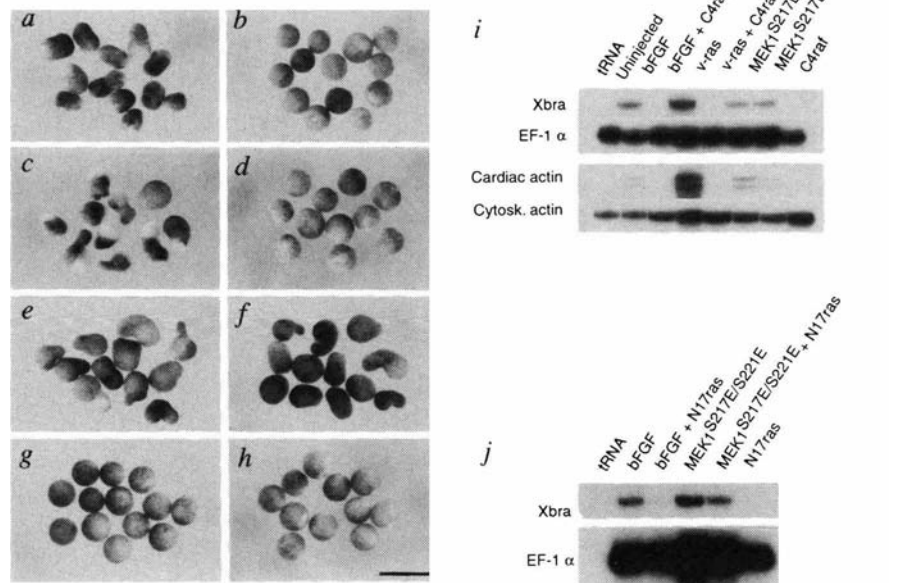
These data indicate that activation of components of the MAP kinase pathway, including MEK1 and *Xenopus* p42, causes ventral mesoderm formation in *Xenopus* embryos. To investigate whether this pathway is necessary for normal mesoderm formation, we studied the effects of a MAP-kinase-specific phosphatase designated X17C (C.S.M. and R.W.O., unpublished observations) on the inducing abilities of FGF, v-ras and MEK1<sup>S217E/S221E</sup> and on the normal development of *Xenopus*.

Figure 3a shows that the presence of X17C RNA inhibits FGF- and MEK1<sup>S217E/S221E</sup>-induced activation of MAP kinase in *Xenopus* animal caps. Animal caps derived from embryos injected with X17C RNA, or from uninjected embryos, were then cultured in the presence or absence of FGF and assayed for elongation and expression of *Xbra*. Caps derived from embryos expressing X17C did not show elongation movements in response to FGF (results not shown) and did not express elevated levels of *Xbra* mRNA (Fig. 3b), indicating that a functional MAP kinase pathway is necessary for mesoderm formation in response to FGF. Additional experiments showed that X17C also inhibits the response to v-ras and MEK1<sup>S217E/S221E</sup> (Fig. 3b).

Intact *Xenopus* embryos injected with X17C RNA developed with characteristic defects. Although the blastopore formed normally, its closure was delayed, and thereafter there was little or

FIG. 4 The mesoderm-inducing effects of an active form of MEK1 are not inhibited by dominant-negative forms of Ras or Raf. *a-h*, Morphological analysis demonstrating that a dominant-negative Raf construct inhibits the functions of bFGF and v-ras but does not affect the action of MEK1<sup>S217E/S221E</sup>. *a, b*, Animal caps treated with 15 U ml<sup>-1</sup> bFGF; *c, d*, animal caps derived from embryos injected with 100 pg p21<sup>v-Ha-ras</sup> RNA; *e, f*, animal caps derived from embryos injected with 2.5 ng MEK1<sup>S217E/S221E</sup> RNA; *g, h*, animal caps derived from uninjected embryos. Animal caps in *b, d, f* and *h* were dissected from embryos that had also been injected with 2.5 ng RNA encoding the C4 dominant-negative form of Raf (ref. 34). Dominant-negative Raf inhibits elongation induced by FGF and v-ras but has little effect on elongation induced by MEK1<sup>S217E/S221E</sup>. *i*, A dominant-negative form of Raf inhibits expression of *Xbra* and of cardiac actin in response to bFGF or v-ras, but does not interfere with the inducing effect of MEK1<sup>S217E/S221E</sup>. *j*, Asn 17 Ras (ref. 11) blocks the ability of FGF to induce expression of *Xbra* but does not inhibit the function of MEK1<sup>S217E/S221E</sup>.

**METHODS.** *Xenopus* embryos were injected with the indicated RNAs as described in the legend to Fig. 1. The C4 dominant-negative form of Raf (ref. 34) was cloned into the vector pSP64T, and RNA was transcribed as described in the legend to Fig. 1. Animal caps were dissected at stage 8, treated where appropriate with bFGF, and cultured to stage 18 for *Xbra* RNase protection analysis, stage 16



for assessment of elongation, or stage 22 for cardiac actin RNase protection analysis. RNase protections with probes specific for *Xbra* or cardiac actin were carried out as described in the legend to Fig. 1.

none of the elongation of the embryo that normally occurs during neurula stages. Analysis of *Xbra* expression at stage 11, and of cardiac actin expression at stage 16, indicated that mesoderm formation in embryos injected with X17C RNA was severely inhibited (Fig. 3c), and when such embryos were reared to larval stages they proved to lack posterior mesodermal derivatives. All displayed a truncated axis posteriorly, with the posterior remnant bent upwards. Intestinal tissue was present. Head development was affected variably but was often remarkably normal (Fig. 3d). This phenotype is similar to that observed when RNA encoding a dominant-negative FGF receptor or a dominant-negative Raf is introduced into early embryos<sup>2,3,10,22</sup>. We conclude that inhibiting MAP kinase activity by overexpressing a MAP kinase-specific phosphatase interferes with posterior mesoderm formation.

Our results show that activation of p42<sup>mapk</sup>, through p21<sup>ras</sup>, Raf and MEK, is necessary and sufficient for induction of ventral mesoderm in *Xenopus* embryos. Ventral mesoderm formation involves characteristic patterns of cell movement, a change in competence to respond to *noggin*, and changes in gene expression, as well as histological differentiation. It is striking that all these properties can be induced solely through the activation of MEK or of p42<sup>mapk</sup>, and that other signalling events associated with the activation of receptor tyrosine kinases or of Ras, such as the activation of PI3-kinase<sup>12</sup>, do not appear to be necessary. To reinforce the conclusion that activation of MAP kinase is sufficient for mesoderm induction we first studied the effects of wortmannin, which inhibits PI3-kinase, and rapamycin, which inhibits activation of p70<sup>S6k</sup>, on the ability of FGF to induce mesoderm. Neither compound blocked induction (results not shown). In additional experiments we overexpressed RNA encoding dominant-negative versions of Ras and Raf, and examined whether these inhibited the mesoderm-inducing activities of MEK1<sup>S217E/S221E</sup>. If the dominant-negative Ras construct inhibits mesoderm formation in response to MEK1<sup>S217E/S221E</sup>, this would suggest that PI3-kinase, or another pathway associated with Ras activation, is also needed for mesoderm formation.

If dominant-negative Raf inhibits MEK1<sup>S217E/S221E</sup>, this would suggest that mesoderm formation requires Raf-dependent activation of targets distinct from MEK. We found that both dominant-negative constructs inhibit mesoderm induction by FGF but neither inhibits the ability of MEK1<sup>S217E/S221E</sup> to induce elongation, to activate mesoderm-specific gene expression, or to undergo terminal differentiation (Fig. 4 and results not shown). This contrasts with the effects of MEK<sup>S217E/S221E</sup> on the transformation of NIH 3T3 cells, where the inhibition of endogenous Ras activity has no effect on the induction of DNA synthesis but does block morphological transformation<sup>15</sup>. Thus, in NIH 3T3 cells cytoskeletal rearrangements are an indirect effect of MEK activity, whereas in *Xenopus* embryos morphogenetic movements are a direct consequence of activation of the MAP kinase pathway.

Together, the results described in this paper show that activation of MAP kinase is the only signal transduction event required for all aspects of FGF-induced ventral mesoderm formation. Of these aspects, one of the most intriguing and poorly understood is that FGF induces different mesodermal cell types at different concentrations<sup>13</sup>, and we note that increasing the activation of the MAP kinase pathway by injection of RNA encoding MEK1<sup>S217E/S221E</sup> has the same effect (Fig. 1i). We are now investigating whether the generation of thresholds in this way is due to different levels of activation of the MAP kinase pathway or to differences in the duration of activation (see ref. 5). □

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## Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice

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THE receptor tyrosine kinase Flk-1 (ref. 1) is believed to play a pivotal role in endothelial development. Expression of the Flk-1 receptor is restricted to endothelial cells and their embryonic precursors<sup>2–5</sup>, and is complementary to that of its ligand, vascular endothelial growth factor (VEGF)<sup>2,3</sup>, which is an endothelial-specific mitogen<sup>6</sup>. Highest levels of *flk-1* expression are observed during embryonic vasculogenesis and angiogenesis<sup>2–5</sup>, and during pathological processes associated with neovascularization, such as tumour angiogenesis<sup>7,8</sup>. Because *flk-1* expression can be detected in presumptive mesodermal yolk-sac blood-island progenitors as early as 7.0 days postcoitum, Flk-1 may mark the putative common embryonic endothelial and haematopoietic precursor, the haemangioblast, and thus may also be involved in early haematopoiesis<sup>4</sup>. Here we report the generation of mice deficient in Flk-1 by disruption of the gene using homologous recombination in embryonic stem (ES) cells. Embryos homozygous for this mutation die *in utero* between 8.5 and 9.5 days post-coitum, as a result of an early defect in the development of haematopoietic and endo-

thelial cells. Yolk-sac blood islands were absent at 7.5 days, organized blood vessels could not be observed in the embryo or yolk sac at any stage, and haematopoietic progenitors were severely reduced. These results indicate that Flk-1 is essential for yolk-sac blood-island formation and vasculogenesis in the mouse embryo.

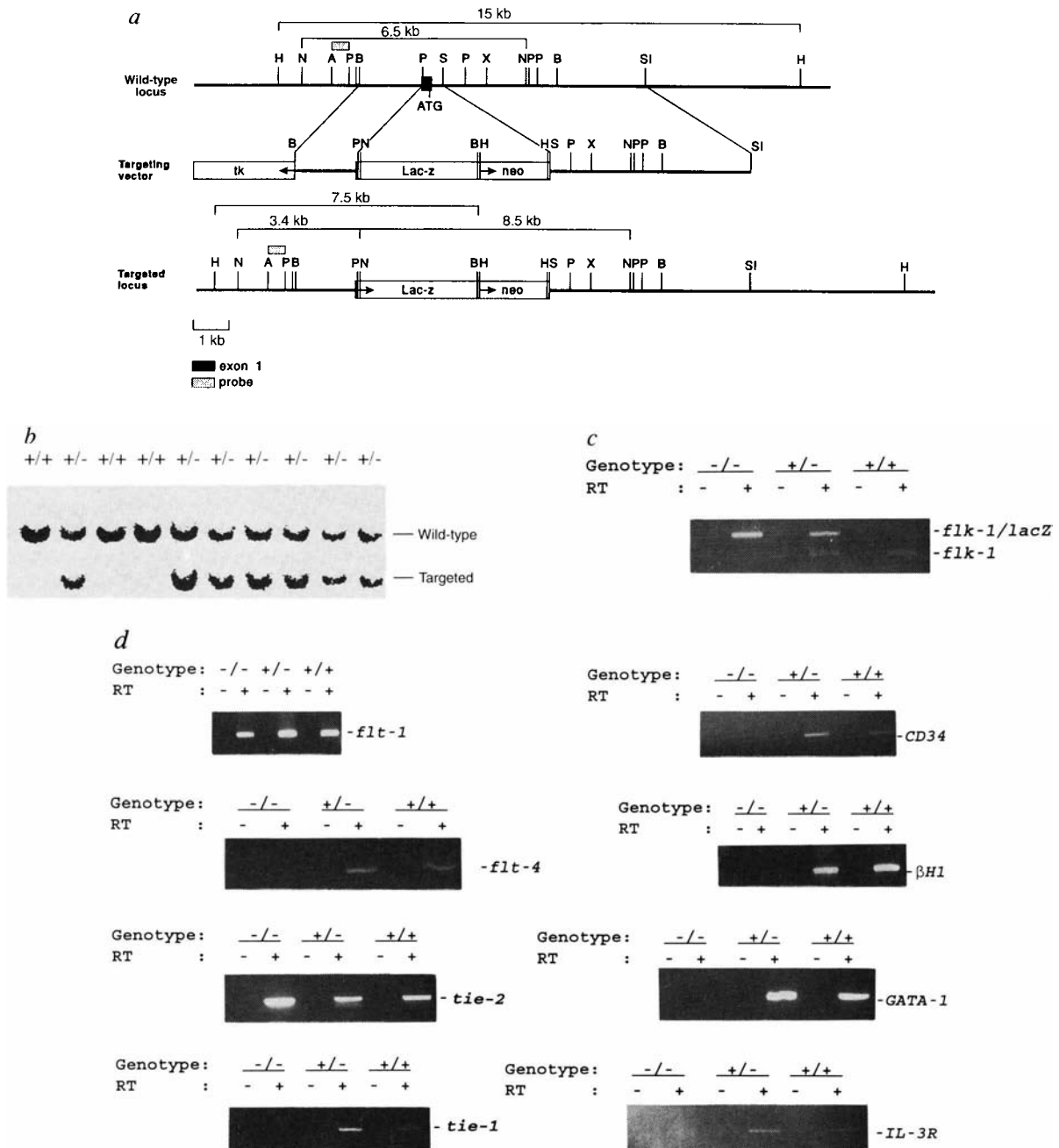
A targeting vector was constructed (Fig. 1a) in which the translated portion of the first coding exon and the proximal part of the next intron of *flk-1* were replaced with a promoterless  $\beta$ -galactosidase gene from *Escherichia coli*. Three independent, correctly targeted ES cell clones transmitted the mutant allele through the germ line of aggregation chimaeras. Heterozygous F<sub>1</sub> and F<sub>2</sub> mice were normal, but no homozygotes were found among 123 newborn animals from heterozygous crosses (Fig. 1b), even though the ratio of wild-type to heterozygous offspring was normal, indicating that the mutation was lethal in the homozygous state.

To determine the time at which the *flk-1* mutation was lethal, embryos from F<sub>1</sub> intercrosses were examined at different developmental stages. Homozygous embryos were mostly resorbed at 10.5 days post-coitum (data not shown), but could still be recovered at 9.5 days (Fig. 2b). At this stage they contained a similar number of somites to, but were slightly smaller than, their heterozygous littermates (Fig. 2a), and had a larger pericardial cavity. The LacZ expression observed in the endocardium, dorsal aortae, intersomitic vessels, small capillaries and yolk-sac vasculature of heterozygous embryos (Fig. 2a, c) could not be detected in the *flk-1*<sup>−/−</sup> homozygotes (Fig. 2b, d), suggesting the absence of these structures. High-level LacZ expression was observed only in the area of the connecting stalk which forms the future umbilical cord and at the position of an aortic arch. Histological examination confirmed the complete absence of organized blood vessels in homozygous embryos and yolk sacs, but also revealed extensive necrosis in the embryo itself, presumably resulting from circulatory failure (data not shown).

Analysis at 8.5 days, before the onset of necrosis, revealed that, in contrast to their heterozygous littermates (Figs 2e and

FIG. 1 Disruption of the *flk-1* gene in mouse embryonic stem cells, and expression of endothelial and haematopoietic markers in *flk-1*<sup>−/−</sup> embryos. a, Restriction maps of mouse *flk-1* genomic fragment, targeting construct, and predicted structure of the targeted *flk-1* allele. The first translated exon is indicated as a solid box. Restriction enzymes: A, *AscI*; B, *BamHI*; H, *HindIII*; N, *NcoI*; P, *PstI*; S, *SmaI*; Sl, *SalI*; X, *XhoI*. Arrows indicate the orientation of *HSV-TK*, *PGK-neo*, and *lacZ* cassettes. Diagonal lines demarcate the areas of homology between the wild-type *flk-1* locus and the targeting vector. Homologous recombination between the targeting vector and the genomic DNA disrupts *flk-1* and places *lacZ* under the transcriptional control of the *flk-1* promoter. Sizes of the expected wild-type and targeted allele *NcoI* and *HindIII* restriction fragments using the external probe indicated by the hatched box are shown. ATG, *flk-1* initiation codon; kb, kilobases. b, Southern blot analysis of tail DNA from an F<sub>1</sub> intercross litter. The 3.4-kb wild-type and 6.5-kb targeted allele *NcoI* fragments identified by the external probe in a are shown; +/+, wild-type, +/−, heterozygote. No homozygous live-born animals were found among 123 offspring analysed. c, Expression of *flk-1* and *flk-1/lacZ* transcripts in wild-type, heterozygous and homozygous 8.5-day embryos. RT-PCR using the primers described below (see methods) produces ~260 base pair (bp) and 135 bp bands specific for *flk-1/lacZ* and *flk-1* which correspond to transcripts arising from the targeted and wild-type alleles, respectively. As expected, homozygous embryos express only the *flk-1/lacZ* fusion transcript and not its wild-type counterpart. d, RT-PCR detection of transcripts corresponding to endothelial receptors Flt-1, Flt-4, Tie-1 and Tie-2, the haematopoietic markers  $\beta$ H1 (embryonic globin expressed during yolk sac erythropoiesis<sup>13</sup>), GATA-1 (a transcription factor essential for primitive and definitive erythroid development<sup>14,15</sup>), and IL-3R (the interleukin-3 receptor, found on early myeloid precursors<sup>16</sup>), and the haematopoietic stem-cell antigen CD34 (ref. 17) is shown. Whereas all markers tested are found in heterozygous and wild-type embryos, only *flt-1*, *flt-4* and *tie-1* transcripts are detected in homozygotes; −/−, homozygote; +/−, heterozygote; +/+, wild-type; RT, reverse

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transcriptase. RT(–) lanes indicate that the observed bands do not arise from genomic DNA.

**METHODS.** Murine *flk-1* genomic clones were isolated from a  $\lambda$ DashII strain 129Sv library (Stratagene), and the position of the first translated exon was mapped by standard procedures<sup>25</sup>. A targeting vector was constructed using a backbone of *pPNTloxP* (a *pPNT*<sup>26</sup> derivative in which two *loxP* sites flank the *PGK-neo* cassette) as follows. The 5' homology region, a 1.8-kb fragment extending from the *Bam*HI site in the upstream region to the *Pst*I site in the untranslated portion of the first coding exon, was inserted into *pSDKlacZ* which contains a promoterless bacterial  $\beta$ -galactosidase gene with an SDK eukaryotic translation initiation sequence and an SV40 polyadenylation signal at its 5' and 3' ends, respectively. The resultant *flk-1-lacZ* fragment was then inserted into the *Bam*HI site of *pPNTloxP*. Finally, a 5.7-kb fragment, which extends from the *Sma*I site about 600 bp downstream of the 3' end of the first coding exon to the *Sal*I site further downstream, was inserted between the *Xho*I and *Not*I sites of the resultant plasmid to form the 3' homology. This final vector was linearized with *Not*I and introduced into R1 ES cells<sup>27</sup> by electroporation. Among ES cell colonies resistant to G418 and gancyclovir, about 1 in 7 contained the expected homologous recombination event, as indicated by Southern blotting using the external probe shown in a and both *Nco*I and *Hind*III. Correct targeting was

confirmed by reprobing the blots with an internal *lacZ* probe (data not shown). For RT-PCR, RNAzol (Tel Test Inc.) was used to extract total RNA from embryos that had been genotyped by Southern blot analysis of RNA prepared from ectoplacental cone and trophoblast cells cultured in ES cell medium<sup>28</sup> without LIF for 10 to 14 days to ensure loss of maternal cells. RNA (5 µg) was reverse transcribed using Superscript reverse transcriptase (BRL) in 20-µl reactions, as recommended by the manufacturer. PCR was then performed as described<sup>29-31</sup> using 0.5 µl portions of cDNA and the primers *lacZ1*, AAAGCGCCATTGCGCATTCa, *Flk-1 F5B*, CTGTGTCCCGCAGCCGGATA (which binds just 5' of the *lacZ* insertion) and *Flk-1 F58*, AAGTCACAGAGCGGGTATGG (which binds just upstream of the *flk-1* translation initiation codon); *Fit1A*, TGTGGA-GAAACTTGGTGACCT and *Fit1B*, TGGAGAACAGCAGGACTCCT (which together yield a 504-bp band); *Fit4A*, CACCGAAGCAGACGCTGATGAT and *Fit4B*, AGCTGCTGTCTCGGAAGAGGT (which yield a 460-bp band); and published *tie-2*, *tie-1* (ref. 29), *βH1*, *GATA-1*, *IL-3R* (ref. 30) and *CD34* (ref. 31) primers. Amplification products were not obtained when reverse transcriptase was omitted. Absence of *flk-1* expression in  $-/-$  embryos was confirmed by western blotting of genotyped 8.5-day embryo lysates using affinity-purified rabbit-*Flk-1* antiserum directed against the carboxy terminus of *Flk-1* (data not shown).



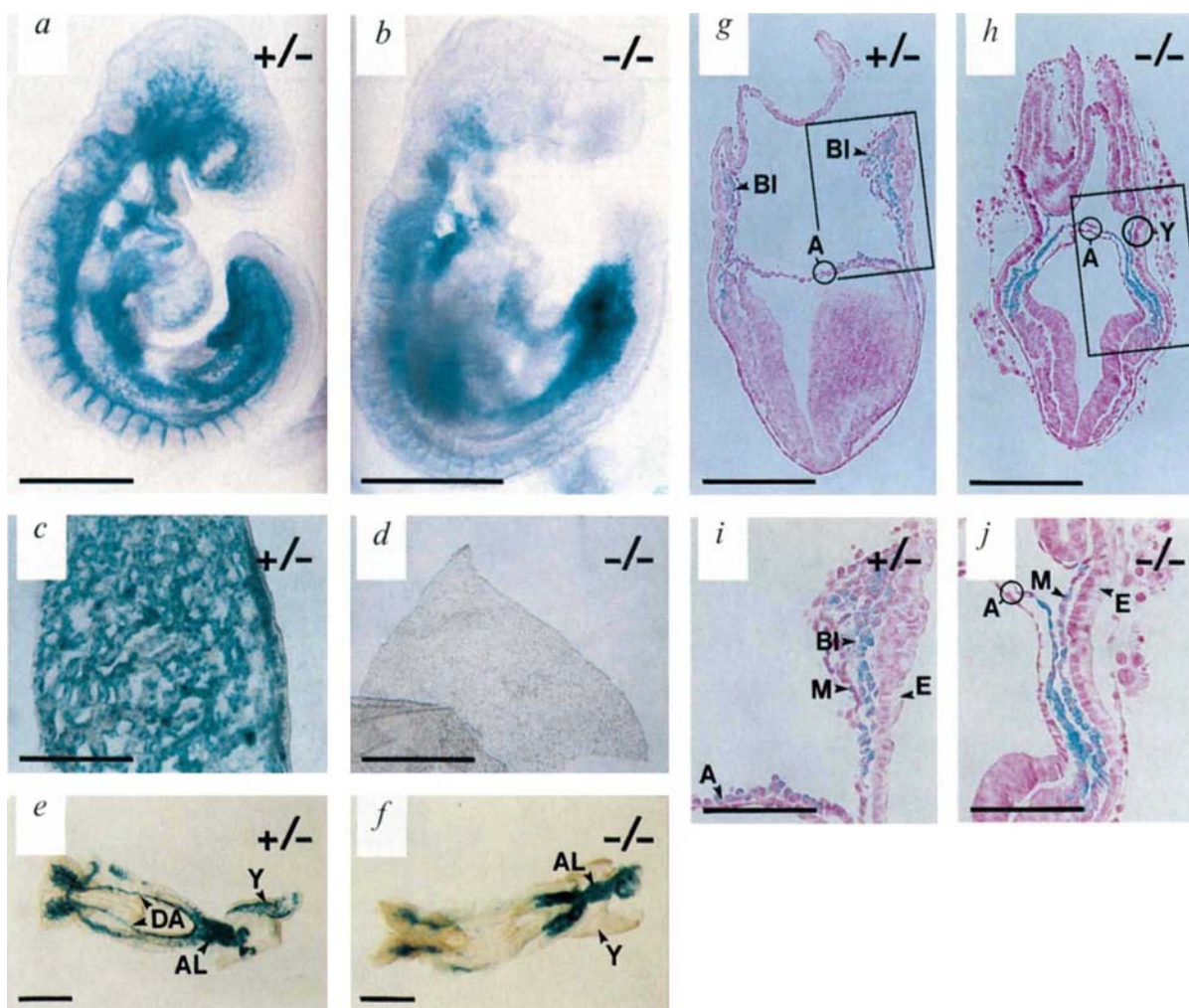


FIG. 2 LacZ expression in *flk-1*<sup>+/-</sup> and *flk-1*<sup>-/-</sup> embryos. **a, b**, Whole-mount 9.5 day post-coitum embryos. LacZ staining in endocardium, dorsal aortae, intersomitic vessels, and capillaries is seen in heterozygotes (**a**) but not in homozygotes (**b**). **c, d**, 9.5-day yolk sacs. The vasculature of heterozygous yolk sacs (**c**) is easily discerned, but is completely absent in homozygotes (**d**). **e, f**, 8.5-day embryos. LacZ-stained vessels are found in *flk-1*<sup>+/-</sup> embryos (**e**), but they are absent in their *flk-1*<sup>-/-</sup> counterparts (**f**). **g–j**, Blood islands do not form in *flk-1*<sup>-/-</sup> embryos. Sagittal sections of 7.5-day LacZ-stained embryos are shown. The LacZ-positive mesenchymal aggregates corresponding to forming blood islands found in heterozygotes (**g, i**) are absent in homozygotes (**h, j**). Scale bar 5  $\mu$ m (**a–f**), 2  $\mu$ m (**g, h**), 1  $\mu$ m (**i, j**). A, amnion; AL, allantois; BI, blood island; DA, dorsal aortae; E, endoderm; M, mesothelium; Y, yolk sac.

3a, c, e, g), homozygous embryos showed no evidence of LacZ-stained blood vessels or endocardium (Figs 2f and 3b, d, f, h). Moreover, homozygous yolk sacs had no blood vessels, and consisted of a single layer of mesothelium lining the yolk-sac endoderm (Fig. 3b, d, f, h). LacZ-positive cells, in which *flk-1* expression has been initiated, were seen in the allantois, the head mesenchyme and flanking the gut, but by electron microscopy did not display mature endothelial cell morphology (data not shown). LacZ expression was also observed at the base of the allantois in heterozygous embryos (Fig. 3a, c), and represents a normal site of *flk-1* expression<sup>3,4</sup>. Expression in the head mesenchyme and other intra-embryonic sites may represent abortive development of endothelial precursors. Indeed, analysis by polymerase chain reaction after reverse transcription of RNA (Fig. 1c, d) indicated that, with the exception of wild-type *flk-1* transcripts which, as expected could not be detected, only markers of early endothelial precursors, that is, *flt-1* (G. Fong, per-

METHODS. Embryos were removed at 9.5, 8.5 and 7.5 days (noon on the day of the vaginal plug is 0.5 days) and were fixed in cold 0.2% glutaraldehyde, 1.5% formaldehyde, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 100 mM sodium phosphate, pH 7.3 for 30 to 45 min. After washing with cold PBS, the embryos were incubated in 2.12 mg ml<sup>-1</sup> potassium ferricyanide, 1.64 mg ml<sup>-1</sup> potassium ferricyanide, 1 mg ml<sup>-1</sup> X-gal, 100 mM sodium phosphate, pH 7.3 overnight at 27°C. Embryos were then washed in PBS and preserved in 3.7% formaldehyde in PBS. For the sections in **g** and **h**, LacZ-stained embryos were embedded in paraffin, sectioned and counterstained with nuclear fast red. Embryos were genotyped by Southern analysis of cultured ectoplacental cone and trophoblast cells as in Fig. 1.

sonal communication), *flt-4* (ref. 9) and *tie-2* (refs 10, 11), were found to be expressed in homozygous embryos, but a marker believed to reflect a slightly later stage of endothelial development, *tie-2* (also called *tek*) (refs 11, 12), could not be detected, indicating the presence of endothelial precursors but not mature cells.

Strikingly, although intravascular blood cells could easily be identified in heterozygous embryos and their yolk sacs at 8.5 days post-coitum, similar cells were not found in homozygotes. In addition, embryos homozygous for the *flk-1* mutation showed no expression of the haematopoietic markers  $\beta$ H1 (ref. 13), *GATA-1* (refs 14, 15) and *IL-3R* (ref. 16) by RT-PCR, although their expression could easily be detected in heterozygotes (Fig. 1d). Moreover, the expression of *CD34*, a marker of primitive haematopoietic stem cells (but which is also expressed in endothelial and other cell types)<sup>17</sup>, was greatly reduced in *flk-1*<sup>-/-</sup> embryos, suggesting that the formation of such cells



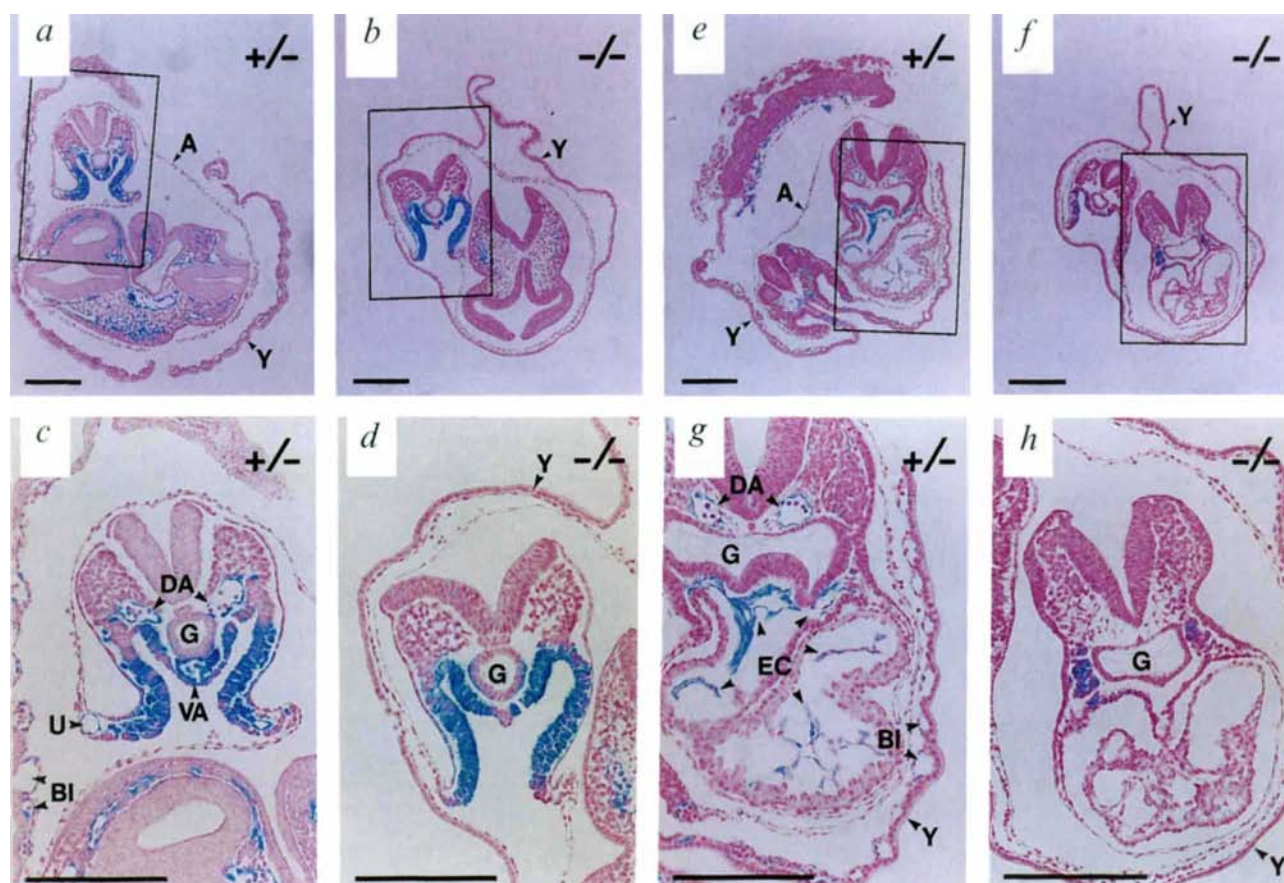


FIG. 3 Absence of blood vessels and blood cells in *flk-1*  $-/-$  embryos. Transverse sections through LacZ-stained 8.5-day  $+/-$  and  $-/-$  embryos are shown at the level of the head and tail (a–d) and, more centrally, through the heart (e–h). The boxed areas in a, b, e and f indicate the regions that are magnified in c, d, g and h, respectively. The dorsal aortae, vitelline artery, and endocardium are found in  $+/-$  (a, c, e, g), but not in  $-/-$  (b, d, f, h), embryos. Homozygous yolk sacs are devoid of blood vessels, and consist of a single layer of mesothelium

lining yolk-sac endoderm (b, d, f, h). Blood cells are seen in heterozygous (c, g) but are absent in homozygous (d, h) embryos. DA, dorsal aorta; VA, vitelline artery; EC, endocardium; A, amnion; G, gut; BI, blood island; U, umbilical vein; Y, yolk sac. Scale bar represents 2  $\mu$ m.

METHODS. Embryos were timed, genotyped, stained for *lacZ* expression, embedded, sectioned and counterstained as in Figs 1 and 2.  $-/-$ , homozygote;  $+/-$ , heterozygote;  $+/+$ , wild-type.

is impaired. Consistent with this observation, only very rare committed haematopoietic progenitors could be found in 8.5-day homozygous yolk sacs by methylcellulose colony assay, whereas they were detectable in large numbers in their heterozygous counterparts (Table 1).

The observed abnormalities of endothelial and haematopoietic development directed our attention to the yolk sac blood islands in which both lineages first arise. At 7.5 days, while *flk-1* expression in heterozygotes was readily detectable in mesenchymal aggregates representing developing blood islands (Fig. 2g, i),

such aggregates were completely absent in homozygotes, and the mesoderm of the yolk sac consisted of a single cell layer only (Fig. 2h, j). Although *flk-1* expression is activated appropriately in the developing mesoderm of mutant embryos, as assessed by the appearance of LacZ staining, cells expressing *lacZ* appear to accumulate in the amnion and yolk-sac mesothelium instead of forming blood islands. We suggest that cells in the posterior primitive streak are signalled to initiate *flk-1* expression but, in the absence of a functional receptor, cannot complete the differentiation pathway to form blood islands, and may be re-directed into other extra-embryonic mesoderm lineages. Continued *lacZ* expression from the *flk-1* promoter in subsets of cells within later embryos, particularly in the base of the allantois and connecting stalk, suggests that there is ongoing induction of *flk-1* expression in these regions. Such cells might normally represent an intra-embryonic source of endothelial<sup>18</sup> and perhaps haematopoietic stem cells<sup>19,20</sup>.

The absence of blood islands and blood vessels in embryos deficient in Flk-1 indicates that the Flk-1 signalling pathway is required very early in the development of the endothelial lineage. Although endothelial precursors may form, mature endothelium does not develop. The absence of haematopoietic precursors suggests that Flk-1 may be important for the development of blood as well, and is consistent with it marking the putative common progenitor of haematopoietic and endothelial cells, the haemangioblast<sup>21</sup>. However, the phenotype of the *flk-1* mutant

TABLE 1 Yolk-sac haematopoietic progenitors in embryos at 8.5 days post-coitum

| Colonies per $+/+$ or $+/-$ yolk sac |                 |                  | Colonies per $-/-$ yolk sac |               |               |
|--------------------------------------|-----------------|------------------|-----------------------------|---------------|---------------|
| Mixed                                | Erythroid       | Myeloid          | Mixed                       | Erythroid     | Myeloid       |
| 33.8 $\pm$ 6.1                       | 60.8 $\pm$ 17.2 | 167.0 $\pm$ 49.2 | 0.5 $\pm$ 0.6               | 1.5 $\pm$ 1.0 | 1.5 $\pm$ 1.3 |

Haematopoietic methylcellulose colony assays were performed using individual yolk sacs treated with 0.25% collagenase/20% fetal calf serum as described<sup>24</sup>. Corresponding embryos were genotyped by Southern blotting (as in Fig. 1). Growth factors added to the methylcellulose included insulin (10  $\mu$ g ml<sup>-1</sup>), erythropoietin (2 U ml<sup>-1</sup>), steel factor (2% conditioned medium), IL-1 $\alpha$  (650 U ml<sup>-1</sup>), IL-3 (100 U ml<sup>-1</sup>), GM-CSF (50 U ml<sup>-1</sup>), and M-CSF (100 U ml<sup>-1</sup>). Colonies (mean  $\pm$  s.d.) were scored on days 7 and 10 of incubation. Under these conditions, colonies are of definitive origin.

embryos is also compatible with a model in which Flk-1 is required for endothelial development, and haematopoietic failure is secondary to the absence of a suitable endothelial-lined microenvironment in the yolk-sac blood islands. We are currently testing these different hypotheses.

The phenotype of mice deficient in Flk-1 is totally different from that of mice lacking the other putative VEGF receptor, Flt-1 (refs 22, 23). This difference indicates that, at least in part, these receptors stimulate different cellular responses, probably by activating different signal transduction pathways. The basis for these distinct responses remains unclear. □

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## Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium

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THE vascular endothelial growth factor (VEGF) and its high-affinity binding receptors, the tyrosine kinases Flt-1 and Flk-1, are thought to be important for the development of embryonic vasculature<sup>1–8</sup>. Here we report that Flt-1 is essential for the organization of embryonic vasculature, but is not essential for endothelial cell differentiation. Mouse embryos homozygous for a targeted mutation in the *flt-1* locus, *flt-1<sup>lcz</sup>*, formed endothelial cells in both embryonic and extra-embryonic regions, but assembled these cells into abnormal vascular channels and died *in utero* at mid-somite stages. At earlier stages, the blood islands of *flt-1<sup>lcz</sup>* homozygotes were abnormal, with angioblasts in the interior as well as on the periphery. We suggest that the Flt-1 signalling pathway may regulate normal endothelial cell–cell or cell–matrix interactions during vascular development.

We generated a mutation in the *flt-1* gene by homologous recombination in embryonic stem (ES) cells<sup>9</sup>. The targeting vector was designed to delete the exon encoding the signal peptide and replace it with the *lacZ* and *neo<sup>r</sup>* genes (Fig. 1a). This is expected to be a null mutation because the start of translation

TABLE 1 Death of *flt-1<sup>lcz</sup>* homozygotes *in utero*

| Stages              | No. tested | +/+ | +/- | -/- |
|---------------------|------------|-----|-----|-----|
| F <sub>2</sub> pups | 119        | 44  | 75  | 0   |
| E11.5               | 34         | 13  | 21  | 0   |
| E9.0                | 38         | 10  | 21  | 7*  |
| E8.0                | 67         | 15  | 31  | 21† |

\* These embryos appeared morphologically equivalent to mid-somite stage embryos (E8.5). The direct cause of embryonic death is not known, although it could be due to the abnormality in the structure and functions of the yolk-sac circulation.

† These embryos were morphologically normal, except that the yolk sacs appeared ruffled. This yolk-sac phenotype was probably a result of the abnormal arrangement of the angioblasts and endothelial cells in the mesodermal layer of the yolk sac.

is removed and reinitiation at the first two downstream Kozak sequences would result in out-of-frame translated products. Targeted ES cell clones were identified by Southern blotting, and three independently targeted ES cell clones were used to generate chimaeric mice by aggregation with CD-1 morula-stage embryos<sup>10</sup>. The targeted allele was transmitted into the germ lines by chimaeras derived from all three ES cell lines (see, for example, Fig. 1b). The heterozygotes were normal and fertile, but the homozygotes had arrested growth at mid-somite stages (E8.5) (Table 1).

The expression of  $\beta$ -galactosidase reflected endothelial cell-specific expression of *flt-1* in heterozygotes and facilitated the analysis of the mutant phenotype. LacZ staining of embryos at early to mid-somite stages demonstrated that, at this stage, *flt-1<sup>lcz</sup>* homozygotes failed to form an organized vascular network in the yolk sac, although LacZ-stained cells were apparent (Fig. 2a, b). Histological sections of the yolk sac revealed that, in heterozygotes, the vascular layer consisted of a series of simple vessels lined by a monolayer of LacZ-expressing endothelial cells. In *flt-1<sup>lcz</sup>* homozygotes, the vascular structures were abnormally large and contained enclosed LacZ-positive endothelial cells as well as the endothelial cells lining the lumen (Fig. 2c, d). Blood cells were found in the enlarged vascular structures. Immunohistostaining with anti-Flk-1 and anti-PECAM-1

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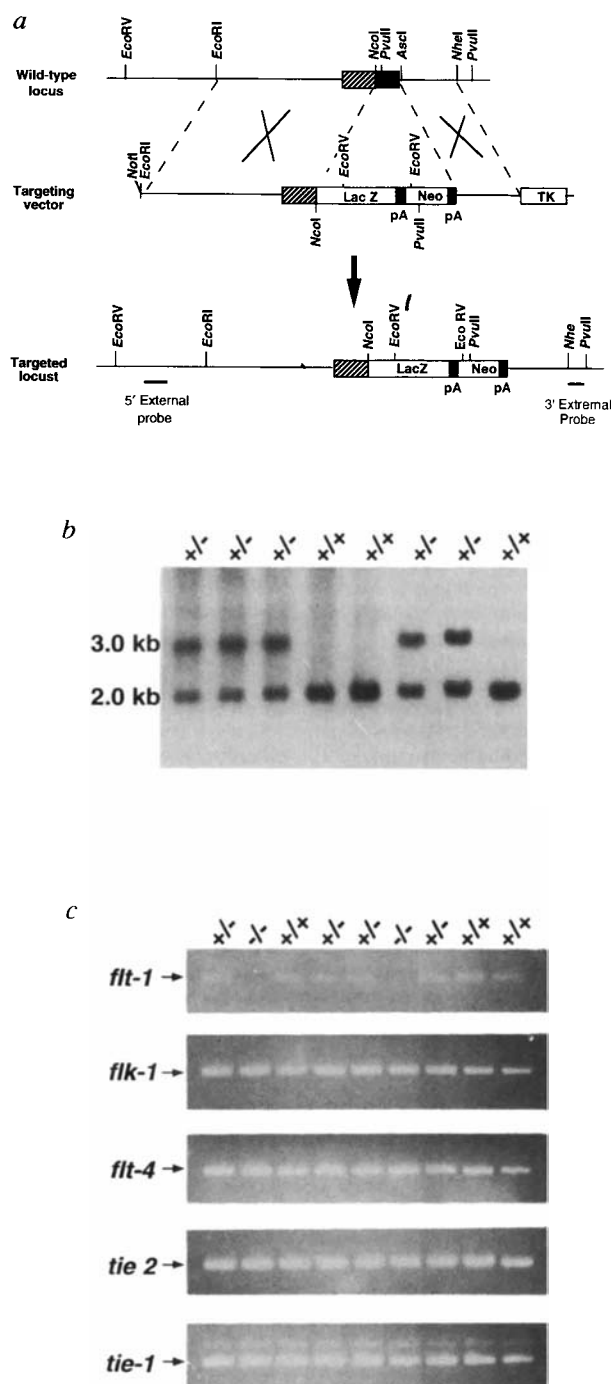
<sup>||</sup> To whom correspondence should be addressed.

<sup>‡</sup> Deceased.

antibodies<sup>11</sup>, which mark differentiated endothelial cells, confirmed that all of the LacZ-expressing cells in the yolk sac of homozygous *flt-1<sup>lacZ</sup>* embryos were of endothelial origin (Fig. 2e–h). These results were further extended by analysis, using polymerase chain reaction after reverse transcription of RNA, of several endothelial receptor tyrosine kinases (RTKs), including Flt-1 (refs 5, 8, 12), Flk-1 (refs 3, 6), Flt-4 (refs 13, 14), Tie-1 (refs 15, 16) and Tie-2 (also known as Tek) (ref. 17) (Fig. 1c). With the exception of Flt-1, all other RTKs were expressed in the homozygous mutants. Thus the genetic basis of endothelial cell formation was not impaired in the *flt-1<sup>lacZ</sup>* homozygotes, but rather, the lack of Flt-1 signalling led to the assembly of endothelial cells into abnormal vascular channels.

Within the embryo itself, similar defects in the organization of the vascular network were observed. In the head mesenchyme,

instead of the progressive development of individual small vessels, large fused vessels were seen which contained internally localized groups of endothelial cells (Fig. 3a, b). The heart and dorsal aortae in the *flt-1<sup>lacZ</sup>* homozygotes appeared to be normal when analysed by whole-mount LacZ staining (Fig. 3c, d, and data not shown). However, histological sections of LacZ-stained embryos revealed that the vascular structures in mutant embryos also displayed phenotypes similar to those observed in the microvasculature of the head and yolk sac. Endothelial cells were found within the dorsal aortic lumen in the *flt-1<sup>lacZ</sup>* homozygotes, but this was not observed in the heterozygotes (Fig. 3e, f). The endocardial lining of the developing heart ventricles was also abnormally thickened and disorganized in homozygotes (Fig. 3g, h).



**FIG. 1** Targeting strategy, germline transmission and RT-PCR analysis. **a**, Gene targeting. To clone the murine homologue of the human *FLT* gene, we designed two oligodeoxynucleotides (ACGCCCGGGCGA-GCAGCCGCGTCGCGCTCACCATG and CCATTATGGGCTGCTCCCCCT-GCA) based on the human *FLT* complementary DNA sequence, and isolated murine *flt-1* cDNA clones by RT-PCR from adult lung RNA. The partial cDNA clone, encoding the 5' coding sequence (164 base pairs) had 83% nucleotide sequence identity to the corresponding region of human *FLT* cDNA, and was later found to be identical to the subsequently reported murine *flt-1* cDNA sequence<sup>21</sup>. Murine genomic clones were isolated from a 129Sv genomic library (from A. Reaume) using this cDNA probe. The genomic clone used for targeting included 12.5 kilobases of upstream sequence, an exon encoding the signal peptide followed by intron sequence. To generate a putative null mutation, an *NcoI*/*Ascl* fragment was replaced by the *lacZ* and *neo'* genes. This resulted in the deletion of the signal peptide coding sequence (shaded box), the splicing donor site, and the insertion of the large *lacZ* and *neo'* cassette containing two polyadenylation sites (filled box and pA). The resulting targeting vector contained 6.5 kb of upstream sequences as the 5' arm, and 1.6 kb of intron sequences as the 3' arm. The 3' external probe detected 2.0 kb and 3.0 kb *PvuII* bands in wild-type and targeted alleles, respectively; the 5' external probe detected a 20-kb (wild-type) and 15-kb (targeted) *EcoRV* bands; the *lacZ* probe detected a single 14-kb *MscI* band from the targeted allele. The homologous recombination events in all three targeted ES cell lines were confirmed by Southern analysis with these probes (data not shown). **b**, Germline transmission of the targeted allele. Tail DNA from a litter of F<sub>1</sub> mice was digested with *PvuII*, and probed with 3' external probe. **c**, RT-PCR analysis of different endothelial markers. Total RNA was purified from a litter of F<sub>2</sub> embryos (E8.5) and used for RT-PCR analysis with oligonucleotides specific for *flt-1* (CGCGTCTTGCTTACGCGCT and CCATTATGGGCTGCTCCCCCTGCA), *flk-1* (AGAACACCAAAAGAGAGGA-ACG and GCACACAGGCAGAAACAGTAG), *flt-4* (CACCAGAGCAGACGC-TGATGAT and AGCTGCTGTCTGCGAAGAAGGT), *tie-1* (ref. 26) and *tie-2* (ACTCTAGAGTCAGAAC-ACACTGCAGAT and AAGACATTCGTTAACACCAC-ACT). As expected, the primers specific for *flt-1* did not detect a PCR product from the homozygotes, whereas all the other PCR primers detected the expected bands. Symbols and abbreviations: hatched box, untranslated region upstream of signal sequence; TK, thymidine kinase gene.



To determine the time of onset of the disorganized vascular phenotype, we examined embryos at late-streak to early head-fold stages, when blood islands are abundant in the yolk sac. Blood-island structures were abnormal in homozygotes (Fig. 2*i, j*); normal blood islands consisted of discrete structures lined by angioblasts and contained haematopoietic progenitors. In contrast, in the homozygotes, angioblasts and haematopoietic cells were intermixed in the disorganized 'blood islands'.

Thus mutation in the *flt-1* gene led to disorganized vascular endothelium from the earliest stages of development, and affected the development of all the examined vascular structures, including major embryonic and extra-embryonic vessels, the endocardium and the microvasculature. The presence of angioblasts in blood islands in homozygotes could mean that haematopoietic precursors were diverted to an angioblastic fate. However, the head mesenchyme, which is probably vascularized by migration of angioblasts from more posterior regions

(angiogenesis)<sup>18</sup>, rather than *in situ* differentiation of mesenchyme cells into endothelial cells (vasculogenesis)<sup>19,20</sup>, displayed a mutant phenotype that is identical to that observed in the yolk sac. Thus it seems unlikely that changes in cell fate determination are involved in the *flt-1* mutant phenotype. The *flt-1* phenotype is also distinct from intussusceptive growth<sup>21</sup>, which also results in the presence of intravascular endothelial cells. Intussusceptive growth primarily occurs during branching of microvessels, whereas the *flt-1* phenotype is seen in the heart, the major vessels and microvessels. Furthermore, the *flt-1* phenotype occurs during formation of blood islands, and so, unlike intussusceptive growth, is independent of previously existing vascular lumens.

Formation of differentiated endothelial cells and their continued proliferation is not affected by the absence of signalling through the Flt-1 receptor. Indeed there may be increased numbers of endothelial cells in the yolk sac and heart endocardium (data not shown), perhaps as a consequence of the failure of

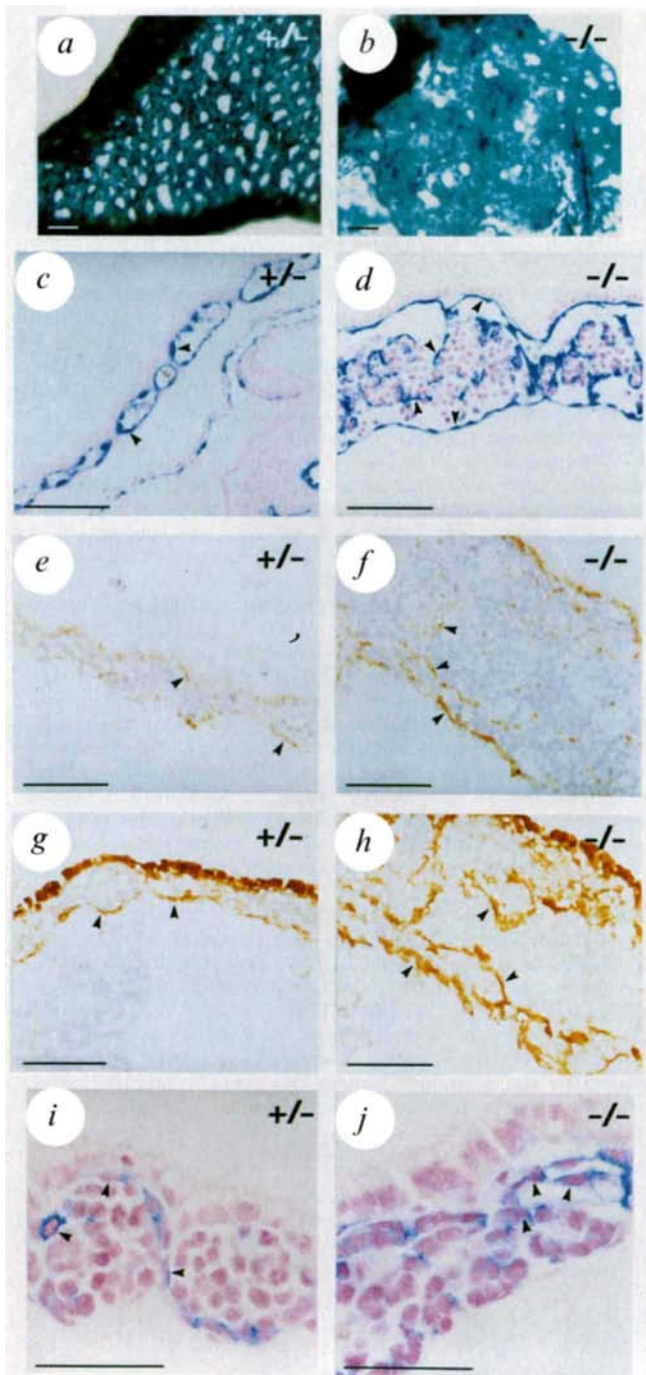
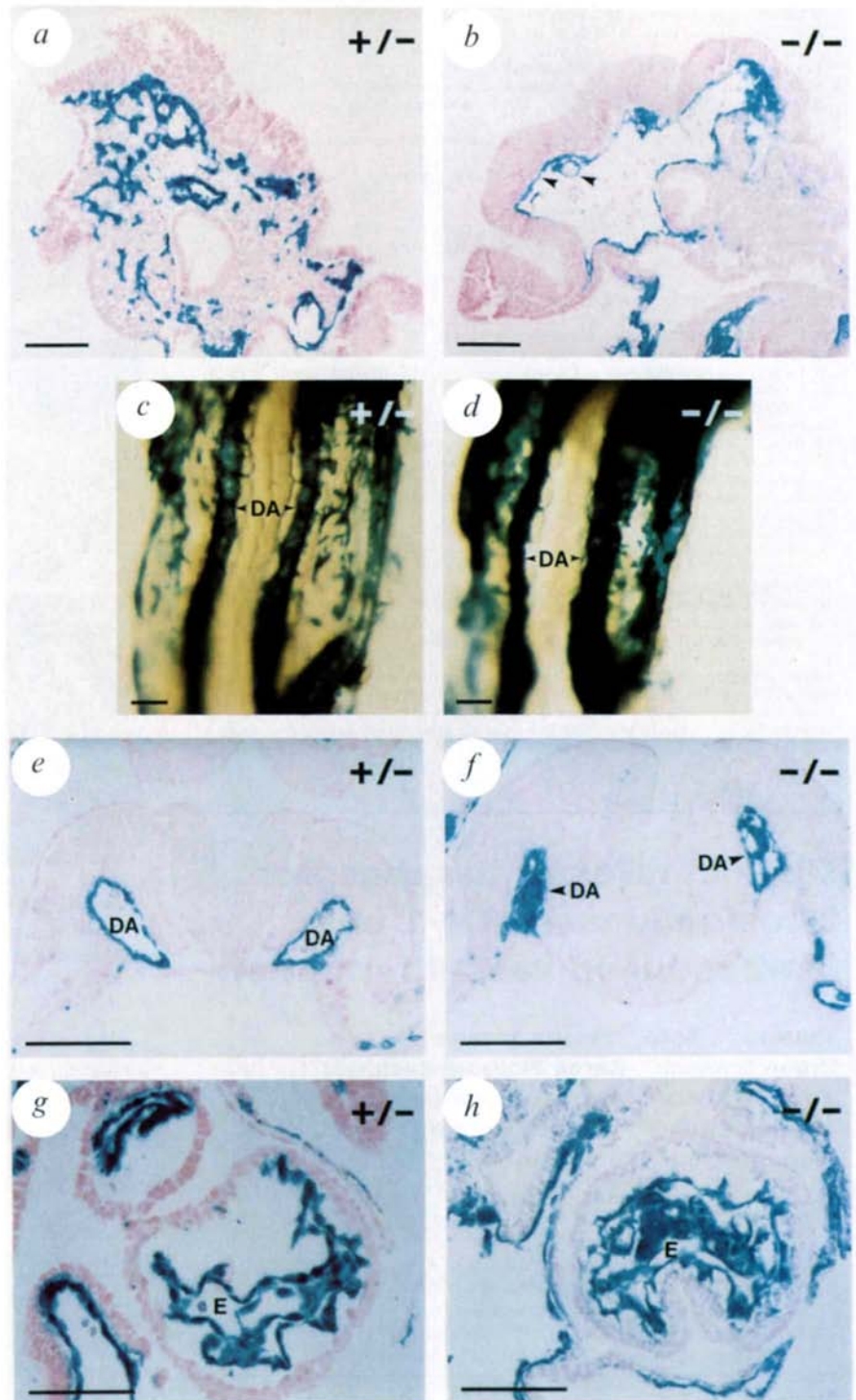


FIG. 2 Disorganized extra-embryonic vasculature in *flt-1<sup>lcz</sup>* homozygotes. *a, b*, Whole-mount lacZ staining of yolk sacs at 8–10 somite stage (E8.5). The heterozygote (*a*) formed the extra-embryonic vascular network by this stage, whereas the homozygote (*b*), although stained blue, lacked obvious vascular structures. *c, d*, Histological sections of lacZ-stained yolk sacs such as those shown in *a* and *b*. In the heterozygote (*c*), endothelial cells (indicated by arrows) lined the normal vascular lumen. In the homozygote (*d*), lacZ-positive cells also displayed elongated endothelial cell morphology (arrows). However, these cells were found both in the lining of lumens and inside the cavities (arrows). In addition, these cells often lined up to form extensive endothelial sheets. *e–h*, Immunohistostaining of normal (*e, g*) and mutant (*f, h*) yolk sacs with anti-Flk-1 (*e, f*) and anti-PECAM-1 (*g, h*) antibodies. In the mutants, as well as in the normal littermate, the elongated cells that were lacZ-positive in similar sections were also positive for both Flk-1 and PECAM-1 expression (indicated by arrows). *i, j*, Sections through lacZ-stained yolk sacs of early head-fold stage embryos (E8.0), showing that *flt-1<sup>lcz</sup>* homozygotes form abnormal blood islands. In heterozygotes (*i*), angioblasts (arrows) were located either at the periphery of a blood island or at the border between adjacent blood islands; in homozygotes (*j*), well-defined blood islands were rarely observed. Instead, angioblasts (arrows) were frequently mixed with haematopoietic cells in the interior of blood islands.

**METHODS.**  $F_1 \times F_1$  matings were set up in the evening and plugs were checked the next morning. Noon of the day a plug was observed was taken as day 0.5 of gestation. Mutant embryos were initially identified by PCR of crude DNA samples prepared from a small piece of yolk sac. After genotyping approximately 100 embryos, we were convinced that homozygotes could be unmistakably identified by a rough yolk-sac phenotype, and so mutants were later identified by this criterion. For whole-mount lacZ staining<sup>27</sup>, dissected embryos and associated extra-embryonic structures were fixed for 30 min at room temperature in fixation buffer containing 0.2% glutaraldehyde, 5 mM EGTA (pH 7.3), 2 mM  $MgCl_2$ , 0.1 M sodium phosphate, pH 7.3. Fixed embryos were then washed a few times with 0.1 M sodium phosphate pH 7.3, and subjected to lacZ staining. Staining reactions were performed for 3 h at 37 °C. The stain contained 2.1  $\mu g\ ml^{-1}$  potassium ferrocyanide (Sigma), 1.6  $\mu g\ ml^{-1}$  potassium ferrocyanide (Sigma), 2 mM  $MgCl_2$ , 1 mg  $ml^{-1}$  X-gal in 0.1 M sodium phosphate, pH 7.3. LacZ-stained embryos were post-fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for at least 12 h at room temperature. Embryos were then embedded in paraffin and sectioned at 6  $\mu m$  thickness. For immunohistostaining, frozen sections were blocked with normal goat serum and avidin-biotin blocking solutions (Vector Laboratories) according to manufacturer's instructions. Sections were then incubated with anti Flk-1 (gift from A. Shih) or anti-PECAM-1 (rat monoclonal antibody Mec 13.3, Pharmingen) under the following conditions: affinity-purified polyclonal rabbit anti-Flk-1 antibody was diluted to 10  $\mu g\ ml^{-1}$  in PBS containing 0.1% Tween 20 and 0.4% SDS (PBST); anti-PECAM-1 antibody was diluted to 10  $\mu g\ ml^{-1}$  in PBS containing 0.1% Triton X100 (PBST). Sections were then incubated with either of these solutions for 1 h at room temperature, and then washed three times with PBST (for anti-Flk-1) or PBST (for anti-PECAM-1). Subsequent incubations with biotinylated secondary antibodies and colour reactions were performed according to manufacturer's instructions.

FIG. 3 Developmental defects in embryonic vasculature. *a, b*, Parasagittal sections through head-fold regions after lacZ staining. The heterozygotes showed numerous cross-sections through microcapillaries, whereas the homozygote formed a large lumen lined by endothelial cells. In addition, endothelial cells were trapped inside the large lumen (arrows). *c, d*, Dorsal view of mid-trunk region of lacZ-stained somite stage 8–10 embryos. The dorsal aortae (DA) of the heterozygote (*c*) and homozygote (*d*) were not obviously different at the whole-mount level. *e, f*, More subtle defects in the dorsal aortae (DA) of *flt-1<sup>lacZ</sup>* homozygotes (*f*) were observed in histological sections. The location of endothelial cells inside the aortic lumen is similar to the phenotype observed in the yolk sac and the head mesenchyme. For comparison, a similar section from a heterozygote is shown in *e*. *g, h*, Endothelial cells were also mislocated in the endocardium of the primitive heart ventricle in *flt-1<sup>lacZ</sup>* homozygotes (*h*). In heterozygotes (*g*), the endocardial cells lined the walls of the forming heart ventricle, in a similar way to endothelial cells lining the vascular lumens of a blood vessel. In the homozygous mutant, extra endothelial cells filled up the ventricular cavity. This is reminiscent of the existence of endothelial cells within vascular lumen of mutant blood vessels. The abnormally located endothelial cells in both dorsal aorta and heart were shown to express PECAM-1 and Flk-1 by immunohistochemistry (data not shown).



contact inhibition of growth in disorganized vessels. The Flt-1 signalling pathway may be important in controlling normal cell adhesion between endothelial progenitor cells, and/or regulating the interaction of endothelial cells with the extracellular matrix. Further analysis of the biological properties of the mutant endothelial cells is required to elucidate the exact downstream pathways affected.

We have also demonstrated<sup>22</sup> that the related receptor, Flk-1, is required for the formation of blood islands, and later blood vessels and haematopoietic cells. Both receptors bind VEGF<sup>1,3</sup> and undergo VEGF-stimulated autophosphorylation<sup>3,23</sup>. Flt-1 also binds placenta growth factor<sup>24</sup>, which is closely related to

VEGF. The distinct phenotypes of embryos lacking either of the two putative VEGF receptors suggest that the downstream response pathways are distinct for the two receptors, and/or that the ligands for the two receptors are not identical *in vivo*.

At least four endothelial-specific RTKs have been identified, including Flk-1, Flt-1, Tie-1 and Tie-2, and mutant phenotypes have now been reported for three of them<sup>22,25</sup>. All of the phenotypes are severe, but affect different aspects or phases of endothelial development. It seems unlikely, therefore, that there is extensive overlap in the functions of these receptors, but rather that multiple signalling pathways are required to execute fully endothelial differentiation and vascularization. □



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## Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation

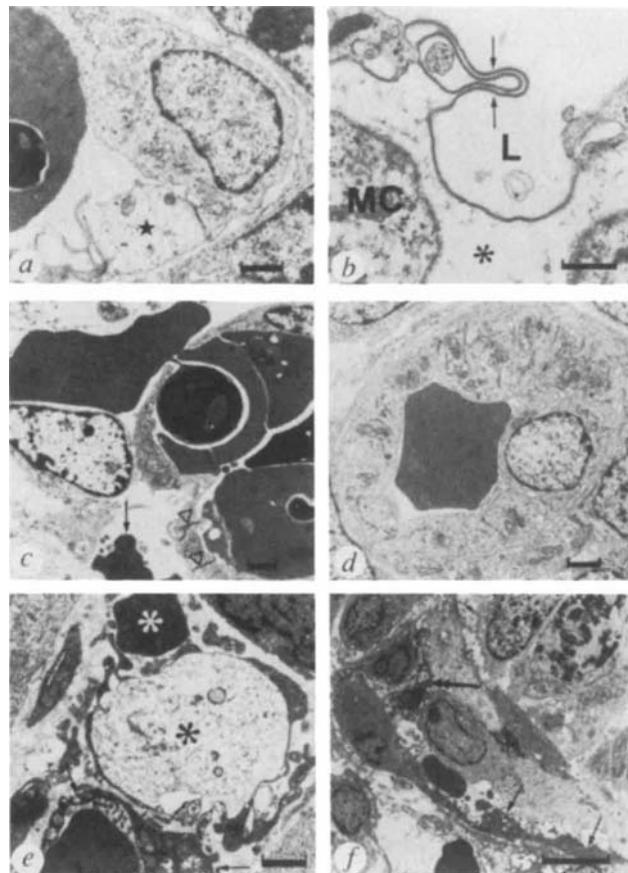
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**TIE-1 and Tie-2 define a new class of receptor tyrosine kinases that are specifically expressed in developing vascular endothelial cells. To study the functions of Tie-1 and Tie-2 during vascular endothelial cell growth and differentiation *in vivo*, targeted mutations of the genes in mice were introduced by homologous recombination. Embryos deficient in Tie-1 failed to establish structural integrity of vascular endothelial cells, resulting in oedema and subsequently localized haemorrhage. However, analyses of embryos deficient in Tie-2 showed that it is important in angiogenesis, particularly for vascular network formation in endothelial cells. This result contrasts with previous reports on Tie-2 function in vasculogenesis and/or endothelial cell survival. Our *in vivo* analyses indicate that the structurally related receptor tyrosine kinases Tie-1 and Tie-2 have important but distinct roles in the formation of blood vessels.**

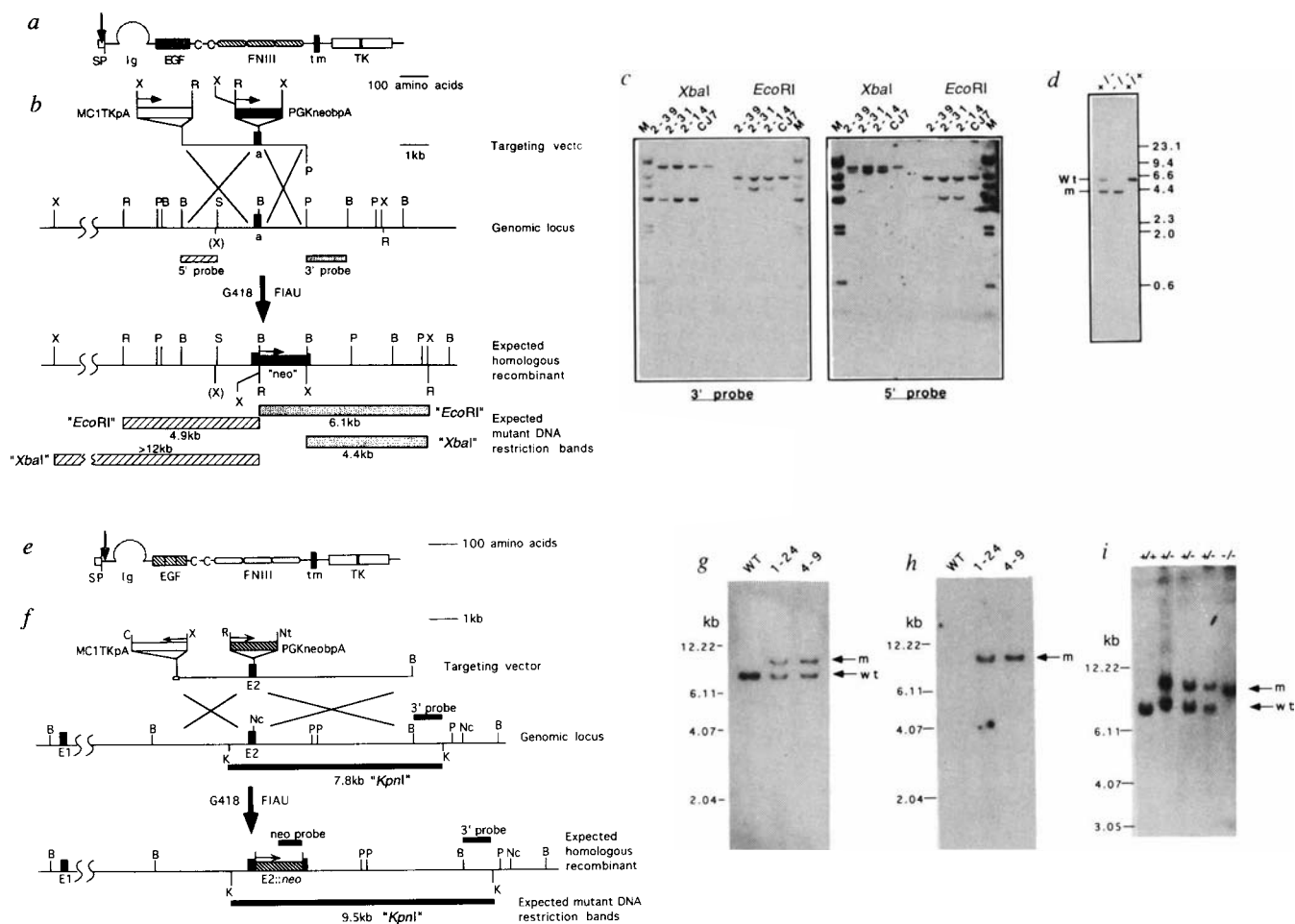


**FIG. 3** Ultrastructural analysis of *tie-1* mutant endothelial morphology. **a**, Submucosa of the stomach of a *tie-1* mutant embryo at E13.5. Star indicates small capillary showing one electron-light endothelial cell. This lightening is interpreted as the first sign of alteration. Scale bar, 1  $\mu$ m. **b**, Atrium endocardium of the heart of a *tie-1* mutant embryo at E13.5. Note the conspicuous, lamellated elongations of endothelial cell membranes (arrows) showing luminal and abluminal membranes closely attached to each other. In the area of these elongations, intercellular junctions are not expressed. MC, cardiac muscle cells; L, lumen; asterisk indicates subendothelial space. Scale bar, 0.5  $\mu$ m. **c**, Tail tip of a *tie-1* mutant embryo at E15.5. Vessels show haemorrhage. The capillaries are extremely full of blood cells, one of which penetrates the capillary wall. The penetration site seems to be outside the junctional complexes, which are well developed (triangles). Arrow indicates free blood cell. Scale bar, 1  $\mu$ m. **d**, Submucosa of the intestine of a *tie-1* wild-type embryo at E15.5. The endothelial cells of a small capillary are intact; haemorrhage is not seen. Scale bar, 1  $\mu$ m. **e**, Intestinal villus of a *tie-1* mutant embryo at E18.5. Lymphatic vessel (black asterisk), arteriole (arrows) and a free blood cell (white asterisk) are shown. Scale bar, 1  $\mu$ m. **f**, Capillary in the intestinal submucosa of a *tie-1* mutant embryo at E18.5. Necrotic endothelial cells (arrow) are directly adjacent to endothelial cells with normal cytoplasm, organelles and junctions. Blood cells just penetrate the necrotic vessel wall (double arrow), others lie extravascularly. Scale bar, 5  $\mu$ m.

**METHODS.** Whole embryos or single organs were fixed by immersion in 1.5% glutaraldehyde/4% paraformaldehyde in PBS, pH 7.4, at 4 °C overnight. Brain, heart, skin, spinal cord, hind limbs and tips of tails were washed again before further processing. All specimens were osmicated in 1% cacodylate-buffered (0.1 M) OsO<sub>4</sub> for 1 h and then dehydrated step by step in ethanol. In 70% ethanol, they were block-stained with saturated uranyl acetate overnight for constant enhancement. Dehydration was completed in absolute ethanol and propyleneoxide. After embedding in Araldite (Serva, Germany), the organs were sectioned on an LKB Nova ultramicrotome. Semithin and ultrathin sections were stained with toluidine blue and lead citrate, respectively. Ultrathin sections were examined on a Zeiss EM 10 electron microscope.

The formation of blood vessels involves sprouting, growth, migration, morphogenic differentiation and remodelling of endothelial cells<sup>1</sup>, which differentiate from the angioblasts present early in development. The differentiation of endothelial cells is accompanied by vasculogenesis<sup>2</sup>, the *in situ* formation of the primary capillary plexus. Subsequent growth and sprouting of endothelial cells results in angiogenesis<sup>2</sup>, the growth and branching of vessels. Molecules that are important in these developmental processes and their regulation are not yet fully explored. Understanding their function and regulation should provide novel diagnostic and therapeutic applications to a variety of pathological conditions, such as tumour angiogenesis and wound healing<sup>3,4</sup>.

Four vascular endothelial cell-specific receptor tyrosine kinases (RTKs) have been isolated and characterized<sup>5-18</sup>. Flk-1 and Flt-1 are receptors for vascular endothelial cell-specific growth factor (VEGF)<sup>10,13,16</sup>. The important role of Tie-2 (also called Tek) receptor tyrosine kinase was shown by dominant-negative and null-mutant transgenic mice<sup>19</sup>. The function of the other endothelial cell-specific RTK, Tie-1, which forms a new class of RTKs with Tie-2 (ref. 15), is not yet known. To study the function of Tie-1 during blood-vessel development, a targeted mutation of the gene was introduced in mice by homologous recombination. Here we show the defects of the developing vascular system *in vivo* caused by the *tie-1* mutation, re-examine



**FIG. 1** Targeted disruption of *tie-1* and *tie-2* genes. **a**, Domain structure of the *tie-1* protein and site of interruption. Tie-1 was interrupted at the second amino acid within the signal peptide sequence. SP, signal peptide; Ig, immunoglobulin domain; EGF, EGF repeats; C, cysteine; FNIII, fibronectin type III repeat; tm, transmembrane domain; TK, tyrosine kinase domains. **b**, Targeting strategy for the *tie-1* gene. The PGKneobpA cassette was inserted at the *Bam*HI site located within the first exon (a) encoding the signal peptide sequences. This vector contains 2.8 kb of homologous sequence upstream of neo and 1.7 kb downstream. An MC1-HSVTKpA cassette was inserted immediately upstream to the 5' *Bam*HI site for negative selection. B, *Bam*HI; X, *Xba*I; R, *Eco*RI; P, *Pst*I. The probes used for hybridization at the bottom. M, labelled lambda *Hind*III size marker. From the top, 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb. Both 2-14 and 2-31 were transmitted through germ line. **d**, Southern blot analysis of embryos derived from *tie-1* heterozygous intercrosses. The genotypes are indicated at the top, and the size markers on the right. DNA was digested with *Xba*I and hybridized with the 3'-flanking probe. The size difference of the wild-type band compared with that in the ES cell Southern blots is due to polymorphism between 129/sv (ES cells) and C57BL/6J (mouse used for crossing) strains. Wt, wild-type; M, mutant. **e**, Domain

structure of the Tie-2 protein and site of interruption. The protein was interrupted immediately after the signal peptide sequence. **f**, Targeting strategy of the *tie-2* gene. The PGKneobpA cassette was inserted at a blunt *Nco*I site in the second exon (E2), and this *Nco*I site is disrupted after the ligation. This vector contains 2.8 kb of homologous sequence upstream from the *Nco*I site and 4.8 kb downstream. The MC1-HSVTKpA cassette was inserted immediately upstream of the 5'-flanking fragment for negative selection. B, *Bam*HI; R, *Eco*RI; K, *Kpn*I; Nc, *Nco*I; P, *Pst*I; X, *Xba*I. **g**, Southern blot analysis of ES cell (1-24, 4-9) DNA with the 3' probe. The size marker is indicated on the left. Both ES cell clones were transmitted through germ line. **h**, Southern blot analysis of ES DNA with the 5' neo probe. **i**, Southern blot analysis of embryos derived from *tie-2* heterozygous intercrosses. DNA samples were digested with *Kpn*I and probed with the 3'-flanking probe. Genotypes are indicated at the top.

**METHODS.** Genomic clones of mouse *tie-1* and *tie-2* genes were isolated from a 129/sv genomic library (Stratagene, San Diego) using mouse complementary DNAs<sup>15</sup>. CJ7 embryonic stem cell culture, electroporation and blastocyst injection were performed as described previously<sup>29</sup>. All heterozygous mice were subsequently crossed to 129/SvEms - + Ter<sup>+</sup>/J (Jackson Lab.) and C57BL/6J (Jackson Lab.) and maintained on both backgrounds. Southern blot analysis was performed as described previously<sup>30</sup>.

the function of Tie-2, and show its important role distinct from that of Tie-1, in blood-vessel formation.

Targeting strategies for *tie-1* and *tie-2* genes, and genotype analyses of embryonic stem (ES) cell clones and mice are summarized in Fig. 1. All neonates homozygous for the *tie-1* mutation ( $n=12$ ) died immediately after birth as a result of breathing difficulties. One day before birth (E18.5), all ( $n=16$ ) homozygous embryos had haemorrhage at the tip of the tail, in several cases, also at the tips of the toes and the sagittal sinus of the head and back, and appeared oedematous subcutaneously (Fig. 2a). Haemorrhage was also observed in internal organs such as intestines and stomach (Fig. 2d). Homozygous embryos also had smaller hearts (Fig. 2c), and histological analysis revealed that alveoli failed to expand in their lungs (Fig. 2e,

f), probably as a result of pulmonary oedema. The intestinal submucosal area of the *tie-1* mutant embryos also exhibited oedema (Fig. 2g, h). When earlier embryos (E13.5) were examined, some of the homozygous embryos (4 of 27, 15%) were found dead and showed the localized haemorrhage (Fig. 2b) seen in older embryos (Fig. 2a). The rest of the mutant embryos (23 of 27, 85%) showed no sign of haemorrhage but exhibited subcutaneous oedema at the back (Fig. 2i). This indicated that the first visible defect in the *tie-1* mutants is oedema, which subsequently develops into localized haemorrhage. These oedematous homozygous embryos had hearts of indistinguishable size from those of heterozygous and wild-type embryos (Fig. 2j). Histological studies of the heart indicated no abnormalities found in other oedematous embryos resulting from abnormal

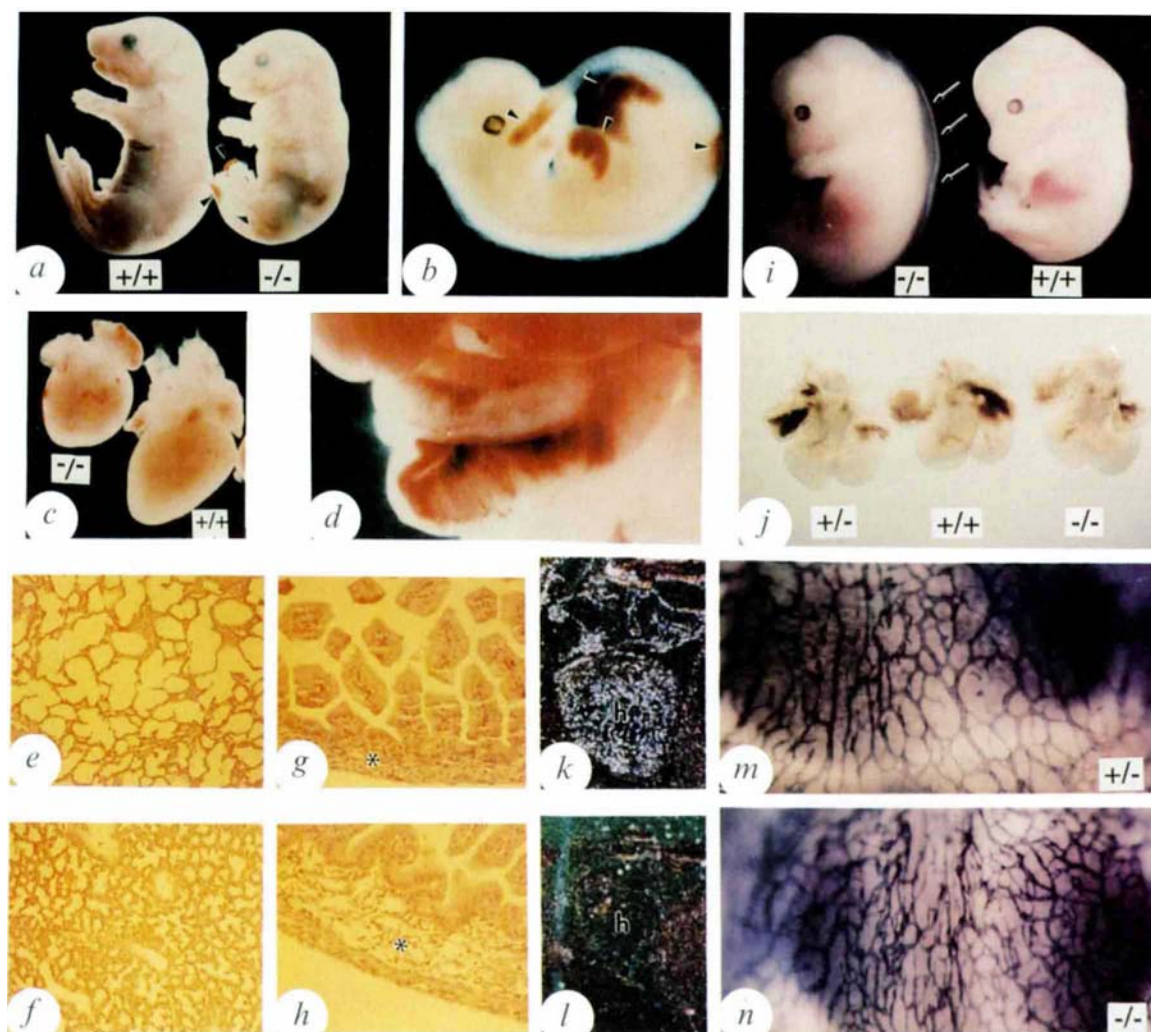


FIG. 2 Phenotypes of Tie-1 homozygous mutant embryos. a, E18.5 embryos. The haemorrhagic tail tip and toes are indicated by arrow heads. b, Some (15%) of the E13.5 embryos were dying and some showed the stereotypic localized haemorrhage (arrow heads). c, E18.5 hearts. d, Haemorrhagic intestines at E18.5. e, f, Sections of wild-type and mutant lungs of E18.5 embryos. Alveoli were fully expanded in the wild type, but failed to expand in the mutants. g, Section of E18.5 intestine from wild-type embryo. Asterisk indicates normal submucosal area. h, Section of E18.5 intestine from mutant embryo. Asterisk indicates oedematous submucosal area. i, E13.5 embryos. Subcutaneous oedema is indicated by arrows. j, E13.5 hearts. k, l, Wild-type and homozygous E13.5 embryo sections hybridized with a Tie-1 riboprobe; h, heart. l, Homozygous E13.5 embryo section hybridized with Tie-1 riboprobe; h, heart. m, n, Vessel patterns of whole-mount heterozygous

and homozygous mutant E12.5 embryos. Skin vasculature over the heart region is shown. The two darker areas underneath are ventricles. The slight increase in vascularization in mutant embryos is variable. METHODS. Paraffin sections, whole-mount staining with anti-PECAM monoclonal antibody and *in situ* hybridization were performed as described previously<sup>25</sup>. Genotypes for embryos were determined by Southern blot using DNA isolated from the yolk sac or a small piece of embryonic tissue as described above. In some cases, PCR genotyping was also performed using the following primers: Tie-1-500, 5'-AGCTGGCCTTGGCTGGCCAG-3'; Tie-1-303R, 5'-GACTTACCAACATGAG-AGGCCAA-3'; neobpA01, 5'-TGGAAGACAATAGCAGGCATGC-3'. The Tie-1-500 and Tie-1-303R pair amplifies a 101-bp wild-type band and neobpA01 and Tie1-303R amplifies a 181-bp recombinant band.



heart structure<sup>20,21</sup>. This suggests that oedema, and subsequent haemorrhage, was not caused by abnormal heart function but by the lack of vessel integrity at the site of leakage. The size difference of the hearts observed in later embryonic stages is probably due to insufficient nutritional supply to cardiac muscles as a result of oedema and haemorrhage. Lack of the mutated *tie-1* gene expression in homozygous embryonic heart was confirmed by *in situ* hybridization (Fig. 2*k, l*). The pattern of the

vascular network of homozygous embryos was studied by staining the E12.5–E13.5 embryos in whole mount with anti-PECAM monoclonal antibody (Fig. 2*m, n*)<sup>22</sup> and was found to have developed a normal vessel pattern (Fig. 2*n*).

The cause of the leaky syndrome manifested by oedema and haemorrhage in *tie-1* mutant embryos was then studied at the ultrastructural level. At the onset of oedema (E13.5), electron-light endothelial cells were detected only in mutant embryos

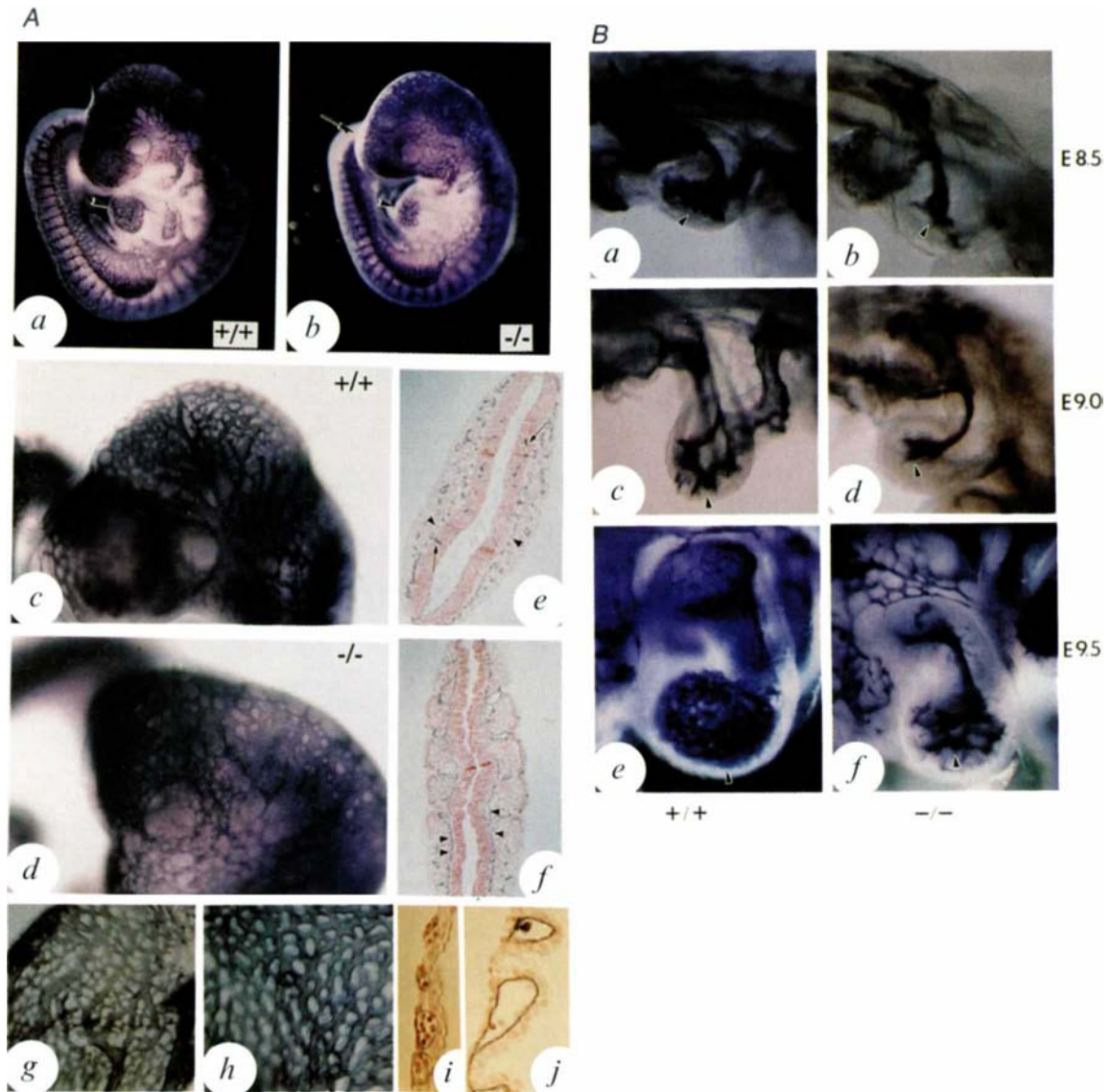


FIG. 4 Phenotypes of *tie-2* homozygous mutant embryos. **A**, Abnormal vascular networks of *tie-2* homozygous mutant embryos. **a, b**, E9.5 embryos stained with anti-PECAM to visualize all the vessel endothelial cells. Homozygous embryo shows growth retardation. Retarded frontal brain (arrow) and heart (arrowheads) are indicated. **c, d**, Higher magnification of head region of E9.5 embryos. Note the lack of large vessels and smaller vessel branches in the homozygous embryo where all the vessels are uniformly dilated. **e**, Transverse section of the wild-type embryonic neural tube. PECAM-positive endothelial cells form tightly closed and compact vessel cavities (examples are indicated by arrow heads) are separated from each other around the neural tube. Examples of capillary sprouts into the neuroectoderm are indicated by arrows. **f**, Transverse section of homozygous mutant neural tube. PECAM-positive endothelial cells form continuous and elongated vessel cavities along the neural tube (examples are indicated by arrow heads) as opposed to the smaller vessels branched off from each other seen in **e**. Note the lack of capillary sprouts into the neuroectoderm in the mutant (**g, h**). **E9.5** wild-type and mutant yolk sacs stained with anti-PECAM in whole mount. **i, j**, Sections of PECAM-stained wild-type and

mutant yolk sacs. **B**, Angiogenic defect of cardiac vessels in *tie-2* homozygous mutant embryos. Heart region of wild-type (**a, c, e**) and mutant (**b, d, f**) embryos (E8.5, E9.0, E9.5) was stained in whole mount with anti-PECAM antibody to visualize growing endothelial cells. Growing branches of the vessels are indicated by arrow heads. Homozygous embryonic vessels failed to form extensive vessel branches at the tips of growing vessels.

**METHODS.** The mid-day of the plug observation was counted as day E0.5. Homozygous and wild-type embryos at the same embryonic stages were confirmed by counting the number of somites. Embryos of the same somite number at each embryonic stage were used for the comparative analyses. The genotypes of dissected embryos were determined by PCR using yolk-sac DNA (for embryo analysis) or embryo DNA (for yolk-sac analysis) as described previously<sup>25</sup>. PCR primers were: T2E2-30, 5'-GGGAATTGATCAAGATCAGGT-3'; T2I5, 5'-TGAGGCTTAGCC-AAGAGGAG-3'. The T2I5 and T2E2-30 pair amplifies a 123-bp wild-type band, and neobpA01 and T2E2-30 pair amplifies a 136-bp recombinant band. The whole-mount staining of embryos and yolk sacs with anti-PECAM monoclonal antibody, MEC13.3, was as described previously<sup>25</sup>.



(Fig. 3a). The heart endocardial cells exhibited a more immature phenotype<sup>23</sup>, such as extreme extension of thin lamellae (Fig. 3b). Although these structures were also found in wild-type hearts, they were detected more frequently in homozygous mutant hearts. Compared with the normal blood vessels (Fig. 3d), study of the site of haemorrhage revealed that erythrocytes extravasate through the blood-vessel endothelial cell, but not between endothelial cells, and that their electron-dense endothelial cell-cell junction structures were intact (Fig. 3c,f). In contrast to blood-vessel endothelial cells, the ultrastructure of lymphatic-vessel endothelial cells was normal (Fig. 3e). These ultrastructural analyses indicate that the development of structural integrity of endothelial cells in homozygous embryos is retarded. This was shown by the presence of morphologically distinct endothelial cells, described above in the mutant embryos. The loss of endothelial integrity then caused leakage of interstitial fluid and subsequently blood through endothelial cells.

In an attempt to investigate the leaky phenotype of *tie-1* mutant endothelial cells *in vitro*, immortalized endothelial cell lines<sup>24</sup> derived from single organs of homozygous and wild-type E18.5 embryos were established. FACS analyses of all cell lines confirmed the quantitatively indistinguishable expression of endothelial markers such as PECAM-1 and MECA32 (ref. 10) (data not shown). An *in vitro* permeability assay showed no difference in <sup>3</sup>H-inulin permeability. Furthermore, preliminary experiments using arterial shear-stress condition (10 dyn cm<sup>-2</sup>) did not reveal any difference (W. Ness and D. Drenckhahn, personal communication). This either indicates that there is loss of endothelial integrity or shows its significance only in an *in vivo* environment involving complex vessel network and hydrodynamics. It is also possible that, owing to the immortalization procedures and/or *in vitro* culture conditions, phenotypic alterations of *tie-1* mutant endothelial cells are no longer detectable.

Previously, *tie-2* function had been analysed using targeted null- and dominant-negative mutation<sup>19</sup>; it was concluded that Tie-2 deficiency results in defects in vasculogenesis and/or survival of endothelial cells. We re-examined the function of Tie-2 using an independently generated, targeted mutant by visualizing all vascular endothelial cells in whole mount with anti-PECAM monoclonal antibody, which has relatively stable expression throughout embryonic development and is ubiquitous in all endothelial cells<sup>22,25</sup>. All *tie-2* homozygous mutant embryos (*n* = 5) were dead at E10.5. Growth retardation, particularly in the head region and the heart, was obvious in all homozygous embryos (*n* = 18) at E9.5 (Fig. 4Aa,b). Close observation revealed a malformation of vascular network. For example, in the head region at E9.5, vessels were uniformly dilated and there was no clear distinction between large vessels and smaller vessels as seen in wild-type embryos (Fig. 4Ac-f), and there were no capillary sprouts into the neuroectoderm in the mutant (Fig. 4Af). Vasodilatation and abnormal vascular network formation of extra-embryonic yolk-sac vessels were also observed (Fig. 4Ag-j). Vessels that form the myocardial circulation failed to form extensive branches (Fig. 4B) beginning at E8.5, indicating that Tie-2 is important in angiogenic processes. The mechanisms, which may be affected, are sprouting and branching (formation of additional vascular cords from the primary capillary plexus) and/or remodelling (conversion of primary capillary plexus to larger and/or smaller vessels). The previous report<sup>19</sup> reached a different conclusion, probably because LacZ expression driven by an incomplete Tie-2 promoter was used as a marker. Because the Tie-2 promoter used only partly recapitulates the endogenous *tie-2* gene expression pattern<sup>24</sup>, and possibly LacZ expression is attenuated by abnormal vascular network formation, the analysis of the complete vascular pattern was obscured.

Deficiency of Tie-1 and Tie-2 *in vivo* resulted in abnormal blood-vessel structure during embryogenesis. However, the phenotypic differences between these embryos revealed distinct functions of these two RTKs. Tie-1 function is related to endothelial cell differentiation and the establishment of blood-vessel

integrity, whereas Tie-2 function is involved in angiogenic processes of endothelial cells.

All four endothelial cell-specific RTKs (Tie-1, Tie-2, Flk-1 and Flt-1) are abundantly expressed during tumour angiogenesis<sup>26,28</sup> (Y.Q., M. Brunda and T.N.S., unpublished results) and normal development. Further study of the signalling mechanisms mediated by these four RTKs is required to decipher the molecular basis of blood-vessel formation during normal development and pathological conditions. This will not only contribute to our basic understanding of molecular mechanisms of blood-vessel formation, but will also be useful for developing novel therapeutic strategies to cure and/or prevent diseases in which blood-vessel formation is important. □

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## Activity-dependent long-term enhancement of transmitter release by presynaptic 3',5'-cyclic GMP in cultured hippocampal neurons

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LONG-TERM potentiation (LTP) in hippocampus is a type of synaptic plasticity that is thought to be involved in learning and memory<sup>1</sup>. Several lines of evidence suggest that LTP involves 3',5'-cyclic GMP (cGMP), perhaps as an activity-dependent presynaptic effector of one or more retrograde messengers (refs 2–12, but

see ref. 13). However, previous results are also consistent with postsynaptic effects of cGMP. This is difficult to test in hippocampal slices, but more rigorous tests are possible in dissociated cell culture<sup>14-17</sup>. We have therefore developed a reliable method for producing *N*-methyl-D-aspartate (NMDA) receptor-dependent LTP at synapses between individual hippocampal pyramidal neurons in culture. We report that inhibitors of guanylyl cyclase or of cGMP-dependent protein kinase block potentiation by either tetanic stimulation or low-frequency stimulation paired with postsynaptic depolarization. Conversely, application of 8-Br-cGMP to the bath or injection of cGMP into the presynaptic neuron produces activity-dependent long-lasting potentiation. The potentiation by cGMP involves an increase in transmitter release that is in part independent of changes in the presynaptic action potential. These results support a presynaptic role for cGMP in LTP.

Induction of LTP in the dentate gyrus *in vivo* or in the CA1 region of hippocampal slices usually requires  $\text{Ca}^{2+}$  influx through postsynaptic NMDA receptor channels<sup>1</sup>. Those channels are normally blocked by  $\text{Mg}^{2+}$  ions, which can be expelled from the channel by depolarization of the postsynaptic neuron. Thus, induction of LTP requires high-frequency (tetanic) stimulation of a number of presynaptic fibres, presumably because stimulation of one or a few presynaptic neurons does not cause sufficient depolarization of the postsynaptic cell<sup>18-20</sup>. Long-lasting potentiation at synapses between single neurons has been produced in culture by removing external  $\text{Mg}^{2+}$  during the tetanic stimulation, but potentiation was seen in relatively few experiments<sup>15</sup>. We have modified the procedure used by altering the electrode solutions and maintaining both neurons under either ruptured or perforated patch whole-cell voltage clamp (instead of current clamp) throughout the experiment, including during the tetanic stimulation (Fig. 1A). The presynaptic neuron was given three high-frequency trains of depolarization (tetani) during brief perfusion with  $\text{Mg}^{2+}$ -free saline, while the postsynaptic neuron remained clamped at the holding potential (to maximize  $\text{Ca}^{2+}$  influx through postsynaptic NMDA receptor channels). This procedure produced an immediate potentiation of the excitatory postsynaptic current (e.p.s.c.) that lasted for the remainder of the experiment (Fig. 1B). In control experiments with no tetanic stimulation or change in  $\text{Mg}^{2+}$  there was usually a gradual decrease in e.p.s.c. amplitude, presumably because of rundown due to the whole-cell patch recording. There was a similar decrease when the bath solution was briefly changed to  $\text{Mg}^{2+}$ -free saline without tetanic stimulation ( $n=4$ ,  $\bar{x}=35\%$  decrease at 25 min, not significantly different from control).

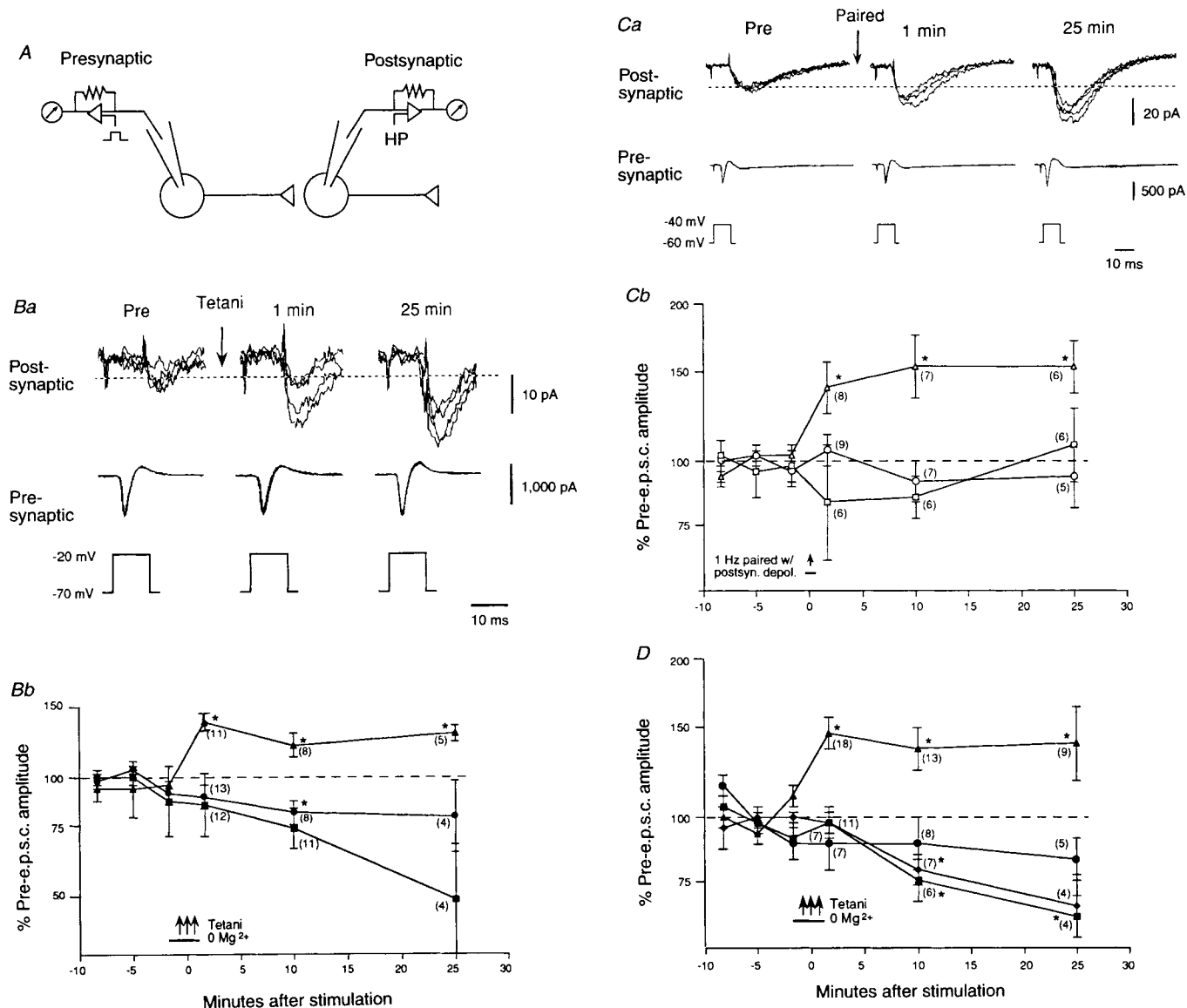
We find that long-lasting potentiation in culture resembles LTP *in vivo* or in slices in several critical ways that indicate that  $\text{Ca}^{2+}$  influx through postsynaptic NMDA receptor channels is required<sup>20-24</sup>. Potentiation at synapses between single neurons in culture was blocked if the cultures were perfused with the NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (APV) (50  $\mu\text{M}$ ) (Fig. 1Bb). Potentiation was also blocked if the tetanic stimulation was delivered in normal  $\text{Mg}^{2+}$  while the postsynaptic neuron either remained clamped at a hyperpolarized level (Fig. 1Bb) or was switched to current clamp during the stimulation ( $n=3$ ,  $\bar{x}=44\%$  decrease 25 min after the stimulation). But we were able to produce long-lasting potentiation in normal  $\text{Mg}^{2+}$  by pairing low-frequency stimulation of the presynaptic neuron (1 Hz for 30 s) with voltage-clamp depolarization of the postsynaptic neuron to 0 mV (Fig. 1C). Like potentiation by tetanic stimulation, potentiation by paired training was blocked by 50  $\mu\text{M}$  APV (Figs 1C and 2Ab). Finally, tetanic stimulation in  $\text{Mg}^{2+}$ -free solution produced no potentiation when the postsynaptic electrode contained the  $\text{Ca}^{2+}$  chelator BAPTA (Fig. 1D). In experiments that were intermixed in the same series, tetanic stimulation without postsynaptic BAPTA did produce significant long-lasting potentiation, replicating the results shown in Fig. 1B.

FIG. 1 Long-lasting potentiation at synapses between individual hippocampal neurons in culture has basic properties of LTP in slices. A, Experimental arrangement. Both the presynaptic and postsynaptic cells were maintained under either ruptured or perforated whole-cell voltage clamp throughout the experiments. B, Long-lasting potentiation by tetanic stimulation and block by APV. Ba, Examples of the e.p.s.c. produced in the postsynaptic neuron by step depolarization of the presynaptic neuron before (Pre), 1 min and 25 min after tetanic stimulation of the presynaptic neuron (three trains at 20-s intervals) during perfusion with  $\text{Mg}^{2+}$ -free solution. The e.p.s.c. was tested once every 50 s. Four successive traces are superimposed at each time period. The dashed line indicates the average Pre value. The current in the presynaptic neuron has had leakage subtracted. On average, the peak inward and peak outward currents in the presynaptic neuron did not change significantly after potentiation in the experiments illustrated in this figure or in C. Bb, Average potentiation by (i) tetanic stimulation in 0  $\text{Mg}^{2+}$  ( $\blacktriangle$ ), (ii) tetanic stimulation in normal  $\text{Mg}^{2+}$  ( $n=5$ ) or APV ( $n=7$ ) ( $\blacksquare$ ) and (iii) test-alone control ( $\bullet$ ). E.p.s.c. amplitude has been normalized to the average value during the 10 min before training (Pre) in each experiment. Average Pre values were (i) 22.9 pA, (ii) 27.5 pA and (iii) 46.8 pA, not significantly different by a one-way analysis of variance (ANOVA). Each point represents the average of four successive trials. Three trains of tetanic stimulation (3 arrows) at 20 Hz ( $n=4$ ) or 50 Hz ( $n=4$ ) for 2 s, or 100 Hz for 1 s ( $n=3$ ), occurred at time zero. The horizontal bar indicates the period during which the bath solution was changed to one with 0  $\text{Mg}^{2+}$ . When APV was used, it was in the bath throughout the experiment. Here and elsewhere the data were log transformed to normalize them for analysis with parametric statistical tests. Averages are therefore reported as geometric means and graphically displayed using log coordinates. Error bars indicate s.e.m. of the transformed data, the numbers in parentheses indicate the  $n$  at each point, and asterisks indicate a significant difference from the Pre level (dashed line). A two-way ANOVA with one repeated measure (test time) revealed that the three training procedures produced significantly different amounts of potentiation ( $F[2, 33 \text{ d.f.}] = 11.38$ ,  $P < 0.01$ ). Subsequent analysis showed that tetanic stimulation produced significantly greater potentiation than each of the other training procedures ( $P < 0.01$  in each case), which were not significantly different from each other. C, Potentiation by low-frequency stimulation of the presynaptic neuron paired with depolarization of the postsynaptic neuron and block by APV. Ca, Example of long-lasting potentiation by paired training. The bath solution contained normal  $\text{Mg}^{2+}$  throughout the experiment. Cb, Average potentiation by (i) paired training ( $\triangle$ ), (ii) paired training in APV ( $\square$ ) and (iii) control ( $\circ$ ). Average Pre values were (i) 20.9 pA, (ii) 19.5 pA and (iii) 47.9 pA, not significantly different by a one-way ANOVA. Stimulation of the presynaptic neuron at 1 Hz for 30 s (arrow) occurred at time zero. The horizontal bar indicates the period (approximately 60 s) during which the postsynaptic neuron was depolarized to 0 mV. When APV was used it was in the bath throughout the experiment. A two-way ANOVA with one repeated measure revealed that the three training procedures produced significantly different amounts of potentiation ( $F[2, 21] = 4.41$ ,  $P < 0.05$ ). Subsequent analysis showed that paired training produced significantly greater potentiation than each of the other training procedures ( $P < 0.05$  in each case), which were not significantly different from each other. D, Long-lasting potentiation by tetanic stimulation is blocked by injection of BAPTA into the postsynaptic neuron. Average potentiation by (i) tetanic stimulation in 0  $\text{Mg}^{2+}$  ( $\blacktriangle$ ), (ii) tetanic stimulation with 20 mM BAPTA in the postsynaptic electrode ( $\blacksquare$ ), (iii) postsynaptic BAPTA without tetanic stimulation ( $\blacklozenge$ ) and (iv) control ( $\bullet$ ). Average Pre values were (i) 30.9 pA, (ii) 33.1 pA, (iii) 114.8 pA and (iv) 46.8 pA, not significantly different by a one-way ANOVA. Tetani were 50 Hz for 2 s. A two-way ANOVA showed that the four training procedures produced significantly different amounts of potentiation ( $F[3, 40] = 14.02$ ,  $P < 0.01$ ). Subsequent analysis showed that tetanic stimulation without BAPTA produced significantly greater potentiation than each of the other training procedures ( $P < 0.01$  in each case), which were not significantly different from each other.

METHODS. Dissociated cell cultures of hippocampal neurons from 1-2-day-old Sprague-Dawley rats were prepared as previously described<sup>17</sup>. After 9-21 days in culture, neurons on a coverslip were transferred to a 1-ml lucite chamber on the stage of an inverted microscope. The bath solution contained (in mM): NaCl (119); KCl (5); HEPES (20);  $\text{CaCl}_2$  (2);  $\text{MgCl}_2$  (2); glucose (30); glycine (0.001); picrotoxin (0.1); pH 7.3, osmolarity adjusted to 330 mosM with sucrose. Tetrodotoxin (TTX, final concentration 2 nM) was added to the bath to reduce spontaneous firing but not completely suppress sodium currents. The bath solution was continuously oxygenated and perfused through the

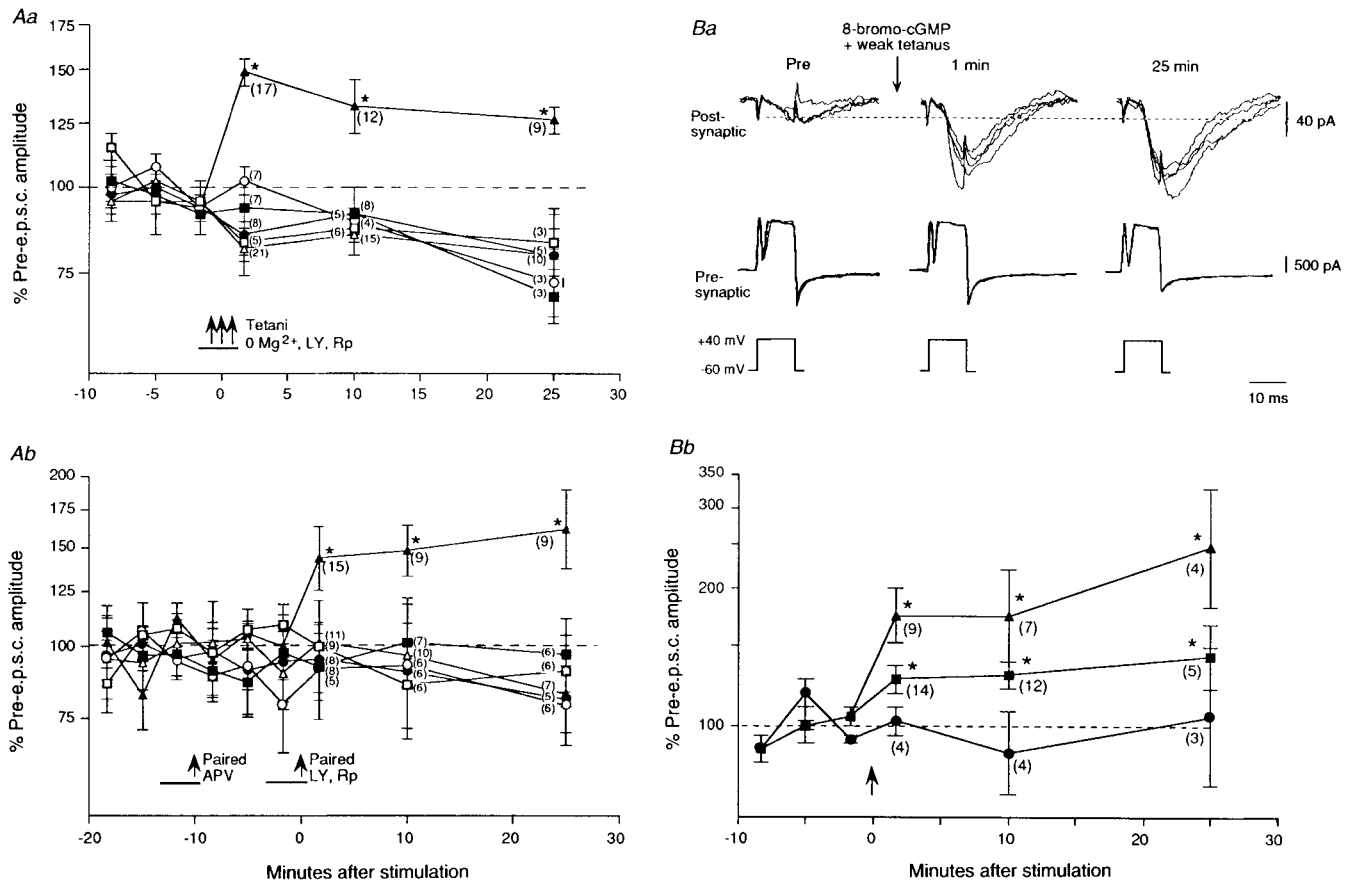
We next examined whether potentiation in culture, like LTP in slices<sup>12</sup>, involves cGMP. Perfusion with a membrane-permeable inhibitor of soluble guanylyl cyclase, LY83583 (0.1 to 1  $\mu$ M), or an inhibitor of cGMP-dependent protein kinase, Rp-8-Br-cGMPs (100  $\mu$ M) completely blocked the induction of potentia-

tion by either tetanic stimulation (Fig. 2Aa) or paired training (Fig. 2Ab), but had no effect on baseline transmission. Conversely, perfusion with a membrane-permeable analogue, 8-Br-cGMP (25  $\mu$ M), for approximately 1 min produced an immediate enhancement of the synaptic current that lasted for the



► chamber at 1 ml min<sup>-1</sup> at room temperature (23–25 °C). Pyramidal-shaped neurons were recorded using either the ruptured (B, D) or perforated (C) whole-cell patch technique under voltage clamp (holding potential -70 mV unless otherwise stated). For ruptured patch recording, the electrode solution contained (in mM): K gluconate (130); KCl (10); MgCl<sub>2</sub> (5); EGTA (0.6); HEPES (5); CaCl<sub>2</sub> (0.06); Mg-ATP (2); GTP (0.2); leupeptine (0.2); phosphocreatine (20); creatine-phosphokinase (50 U/ml). Seals of G $\Omega$  resistance were obtained on a pair of cells and access to the cells was obtained by application of a brisk negative pressure to the seal. For perforated patch recording, the tip of the patch electrode was filled with a solution containing (in mM): K<sub>2</sub>SO<sub>4</sub> (75); KCl (10); HEPES (10); pH 7.2, osmolarity adjusted to 310 mosM with sucrose. The rest of the electrode was back-filled with the same solution plus 300  $\mu$ g ml<sup>-1</sup> amphotericin B diluted from a stock of 100 mg amphotericin B per 100  $\mu$ l DMSO. Recording pipettes had resistances of 3–5 M $\Omega$  with these solutions. Seals of G $\Omega$  resistance were obtained on a pair of cells, and the access resistances were monitored until they stabilized (generally after 4–15 min) before an experiment was begun. The access resistances were checked for constancy throughout all experiments, and capacitance compensation was used. Finding synaptically connected pairs of neurons was easier if neurons were maintained

for more than 10 days in culture. The cell bodies were usually 10–100  $\mu$ m apart, with synaptic contacts distributed on the cell bodies and along processes growing between them (D. Baranes, personal communication). The presynaptic neuron was stimulated with a 10-ms positive voltage step, sufficient to elicit a large inward current (which probably represents an unclamped action potential in the distal processes) in the presynaptic neuron, and an e.p.s.c. in the postsynaptic neuron. Several lines of evidence suggest that the e.p.s.cs were monosynaptic: (1) they had short and fairly constant latencies (on average less than 3 ms), (2) they followed presynaptic depolarizations one-for-one at high frequencies (up to 100 Hz) even in bath solutions containing elevated concentrations of divalent cations (5 mM Ca<sup>2+</sup>, 10 mM Mg<sup>2+</sup>) and (3) the e.p.s.cs did not have multiple peaks. Signals were recorded with an Axopatch 1A and an Axopatch 200, filtered at 1 kHz and analysed on a microcomputer using the P-clamp program from Axon Instruments. E.p.s.c. amplitudes were measured between the peak and the mean of the baseline just before the start of the e.p.s.c. All chemicals were from Sigma except for BAPTA tetrapotassium salt (Molecular Probes, Eugene, OR), LY83583 (Biomol, Plymouth Meeting, PA), and Rp-8-Br-cGMPs (Biolog, La Jolla, CA).



**FIG. 2** Long-lasting potentiation in culture involves cGMP. **A**, Long-lasting potentiation is blocked by inhibitors of guanylyl cyclase (LY83583) or cGMP-dependent protein kinase (Rp-8-Br-cGMPs). **Aa**, Potentiation by tetanic stimulation. Average enhancement of e.p.s.c. amplitude by (i) tetanic stimulation in 0 Mg<sup>2+</sup> (▲), (ii) control (△), (iii) tetanic stimulation in 0 Mg<sup>2+</sup> and LY83583 (●), (iv) 0 Mg<sup>2+</sup> and LY83583 alone (○), (v) tetanic stimulation in 0 Mg<sup>2+</sup> and Rp-8-Br-cGMPs (■) and (vi) 0 Mg<sup>2+</sup> and Rp-8-Br-cGMPs alone (□). Experiments in (i) and (ii) were intermixed with the other experiments, and include experiments from Fig. 1Bb and experiments in which vehicle was injected into either the pre-synaptic or postsynaptic neuron (see Fig. 3 legend). Tetani in these experiments were 50 Hz for 2 s. Average Pre values were (i) 19.1 pA, (ii) 31.6 pA, (iii) 49.0 pA, (iv) 34.7 pA, (v) 72.4 pA and (vi) 45.7 pA, not significantly different by a one-way ANOVA. Both the presynaptic and postsynaptic cells were recorded using the ruptured whole-cell patch technique in these experiments. The horizontal bar indicates the period during which the bath solution was changed to one with 0 Mg<sup>2+</sup>, 0 Mg<sup>2+</sup> and LY83583, or 0 Mg<sup>2+</sup> and Rp-8-Br-cGMPs. A two-way ANOVA with one repeated measure (test time) revealed that the six training procedures produced significantly different amounts of potentiation ( $F[5, 64] = 5.60$ ,  $P < 0.01$ ). Subsequent analysis showed that tetanic stimulation produced significantly greater potentiation than each of the other training procedures ( $P < 0.01$  in each case), none of which was significantly different from any other. **Ab**, Potentiation by low-frequency stimulation (1 Hz for 30 s) paired with depolarization of the postsynaptic neuron to 0 mV. Average enhancement of e.p.s.c. amplitude by (i) paired training (▲), (ii) control (△), (iii) paired training in LY83583 (●), (iv) LY83583 alone (○), (v) paired training in Rp-8-Br-cGMPs (■) and (vi) Rp-8-Br-cGMPs alone (□). Average Pre values were (i) 18.2 pA, (ii) 40.7 pA, (iii) 43.7 pA, (iv) 49.0 pA, (v) 30.9 pA and (vi) 57.5 pA, not significantly different by a one-way ANOVA. Both the presynaptic and postsynaptic cells were recorded using the perforated patch technique in these experiments. The first horizontal bar indicates the period during which the bath solution was changed to one with APV (50 μM). Paired

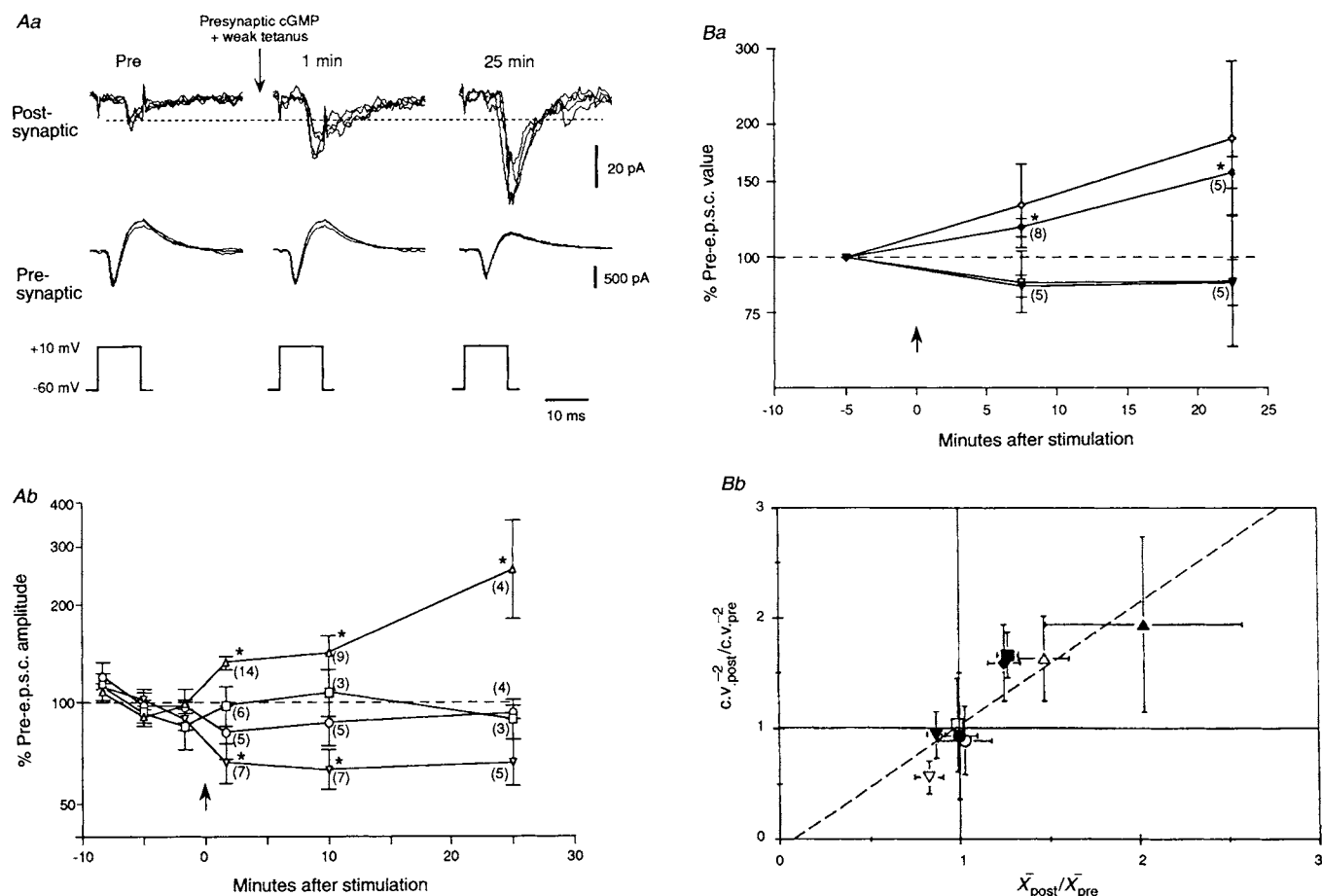
training during perfusion with APV did not produce any potentiation, replicating the results shown in Fig. 1Cb. The APV was then washed out, and the second horizontal bar indicates the period during which the bath solution was changed to one with normal saline, LY83583, or Rp-8-Br-cGMPs. A two-way ANOVA with two repeated measures showed that paired training in normal saline produced significantly greater potentiation than paired training in APV in the same cells ( $F[1, 14] = 23.79$ ,  $P < 0.01$ ). Two-way ANOVAs with one repeated measure showed that paired training in normal saline also produced significantly greater potentiation than each of the five other training procedures ( $P < 0.05$  in each case), none of which was significantly different from any other. **B**, Activity-dependent long-lasting enhancement of e.p.s.c. amplitude by 8-Br-cGMP. **Ba**, Example of enhancement by brief (30–60 s) bath application of 25 μM 8-Br-cGMP at the same time as weak tetanic stimulation of the presynaptic neuron (a 50-Hz, 0.5-s train of 10-ms positive-voltage steps eliciting inward currents in the presynaptic neuron) ('paired' training). Both the presynaptic and postsynaptic cells were recorded using the whole-cell perforated patch technique in these experiments. The current in the presynaptic neuron has not had leakage subtracted in this example. We did not see any consistent change in the inward or outward currents in the presynaptic neuron or the holding or leakage currents in the postsynaptic neuron during or after the training procedures illustrated in this figure or in Fig. 3. **Bb**, Average time course of enhancement by (i) 8-Br-cGMP paired with weak tetanus (▲), (ii) 8-Br-cGMP alone with ( $n = 6$ ) or without ( $n = 8$ ) 50 μM APV in the bath (results were similar and have been pooled (■)), and (iii) weak tetanus alone (●). Average Pre values were (i) 33.1 pA, (ii) 46.2 pA and (iii) 28.3 pA, not significantly different by a one-way ANOVA. A two-way ANOVA with one repeated measure revealed that the three training procedures produced significantly different amounts of potentiation ( $F[2, 24] = 9.18$ ,  $P < 0.01$ ). Subsequent analysis showed that paired training produced significantly greater potentiation than training with 8-Br-cGMP alone or weak tetanus alone ( $P < 0.05$  in each case).



remainder of the experiment (Fig. 2*Bb*). To investigate whether potentiation by 8-Br-cGMP in culture, as in slices<sup>12</sup>, is enhanced by paired spike activity, we produced a weak tetanus (50 Hz, 0.5-s train of depolarizations) in the presynaptic neuron during application of the 8-Br-cGMP ('paired' training) (Fig. 2*Ba*). These experiments were conducted in normal  $Mg^{2+}$  and 50  $\mu M$  APV to ensure that training with the tetanic stimulation alone

did not produce potentiation. None the less, paired training produced significantly greater potentiation than 8-Br-cGMP alone (Fig. 2*Bb*).

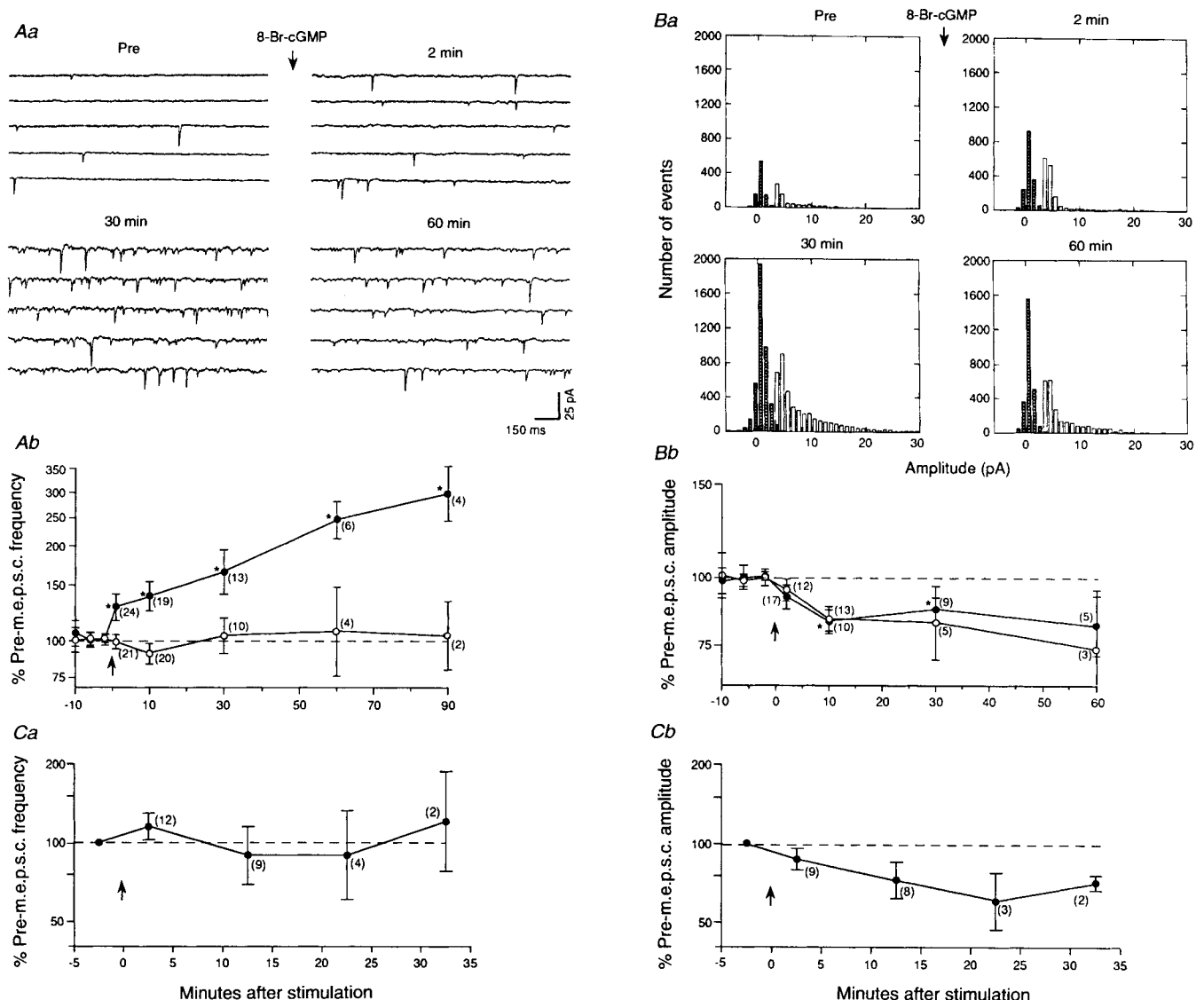
Because 8-Br-cGMP is membrane permeable, its site of action could be in either the presynaptic or postsynaptic cell. To distinguish between these possibilities we injected cGMP into one cell or the other through the recording electrode, either paired or



**FIG. 3** Presynaptic cGMP produces enhancement of e.p.s.c. amplitude and  $c.v.^{-2}$ . **A**, Activity-dependent long-lasting enhancement of e.p.s.c. amplitude by presynaptic injection of cGMP. **Aa**, Examples of the e.p.s.c. before (Pre), 1 min and 25 min after presynaptic injection of cGMP paired with weak tetanic stimulation of the presynaptic neuron. The current in the presynaptic cell has had leakage subtracted in this example. **Ab**, Average time course of enhancement by (i) presynaptic injection of cGMP paired with weak tetanus ( $\blacktriangle$ ), (ii) presynaptic control injection paired with weak tetanus ( $\circ$ ), (iii) presynaptic cGMP alone ( $\square$ ), and (iv) postsynaptic cGMP paired with weak tetanus ( $\nabla$ ). Average Pre values were (i) 23.4 pA, (ii) 18.2 pA, (iii) 29.5 pA and (iv) 20.4 pA, not significantly different by a one-way ANOVA. A two-way ANOVA revealed that the four training procedures produced significantly different amounts of potentiation ( $F[3, 29] = 21.58$ ,  $P < 0.01$ ). Subsequent analysis showed that presynaptic cGMP paired with tetanus produced significantly greater potentiation than each of the other training procedures ( $P < 0.01$  in each case). **B**, Comparison of changes in e.p.s.c. amplitude and  $c.v.^{-2}$ . **Ba**, Time course of changes in e.p.s.c. amplitude (filled symbols) and  $c.v.^{-2}$  (open symbols) after (i) presynaptic injection of cGMP paired with weak tetanus ( $\blacktriangle$ ), and (ii) postsynaptic injection of cGMP paired with weak tetanus ( $\blacktriangledown$ ). In these experiments the e.p.s.c. amplitude was tested once every 10 s. The mean and  $c.v.^{-2}$  were calculated for 15-min intervals (40–90 events) and normalized to the average value during the 10 min before training (Pre) for each experiment. Average Pre values were (i) 51.3 pA and (ii) 6.8 pA. A two-way ANOVA revealed that the two training procedures produced significantly differ-

ent amounts of potentiation ( $F[1, 11] = 24.67$ ,  $P < 0.01$ ), replicating the results shown in **Ab**. **Bb**, Average ratio of  $c.v.^{-2}$  during the 10 min before training (Pre) and up to 25 min after training (Post), plotted against the average ratio of mean e.p.s.c. amplitude ( $\bar{x}$ ) in the experiments in **Ba** and those in Figs 2*Bb* and 3*Ab* with at least 8 post-tests. Although they were calculated from fewer events, the average results from the experiments in **Ab** were similar to those in **Ba**. Symbols are the same as in Figs 2*Bb*, 3*Ab* and 3*Ba*. The dashed line indicates the linear regression fitted to the plotted points. The average values for  $c.v.^{-2}$  before training were: Fig. 2*Bb*, (i) 19.2, (ii) 27.6 and (iii) 26.7, not significantly different by a one-way ANOVA; Fig. 3*Ab*, (i) 16.9, (ii) 6.3, (iii) 7.6 and (iv) 10.8, not significantly different; Fig. 3*Ba*, (i) 14.5 and (ii) 2.8, not significantly different.

**METHODS.** Ruptured whole-cell patch recording was used for both cells. Drugs were applied intracellularly by means of a fast internal perfusion method<sup>28</sup>. A perfluoroethylene (PEF) tube was pulled to a very fine diameter, filled with the electrode solution and the drug, and inserted to within 300  $\mu m$  of the tip of the electrode. At the time of injection, a motorized micrometer spindle began pushing a Hamilton microtitre syringe filled with Nujol mineral oil and connected to the PEF tubing. 2 min after the beginning of injection at a rate of 2  $\mu l \text{ min}^{-1}$ , weak tetanic stimulation was delivered to the presynaptic neuron. The injection was stopped immediately after the end of the tetanic stimulation, allowing the drug to be diluted in the much larger volume of the electrode ( $\sim 80 \mu l$ ).



**FIG. 4** Effects of 8-Br-cGMP and postsynaptic injection of cGMP on spontaneous m.e.p.s.cs. **A**, Long-lasting enhancement of spontaneous m.e.p.s.c. frequency by 8-Br-cGMP. **Aa**, Examples of spontaneous m.e.p.s.cs before (Pre), 2 min, 30 min and 60 min after brief application of 100  $\mu$ M 8-Br-cGMP. **Ab**, Average time course of enhancement. 8-Br-cGMP ( $\bullet$ ) or normal saline ( $\circ$ ) was applied at time zero (arrow). M.e.p.s.c. frequency has been normalized to the average value during the 12 min before the application (Pre) in each experiment (average = 60.3  $\text{min}^{-1}$  for 8-Br-cGMP and 55.0  $\text{min}^{-1}$  for control, not significantly different). Each point represents the average during a 1 to 4-min period. The increase in m.e.p.s.c. frequency after 8-Br-cGMP application was significantly greater than after normal saline perfusion ( $P < 0.05$  by *t*-test at each time point). **B**, No enhancement of m.e.p.s.c. amplitudes by 8-Br-cGMP. **Ba**, Amplitude histograms of spontaneous m.e.p.s.cs (open bars) before (Pre), 2 min, 30 min and 60 min after brief application of 8-Br-cGMP in the experiment illustrated in **Aa**. The threshold for m.e.p.s.c. detection in this experiment was set at 3 pA. The hatched bars show the distribution of the recording noise, measured 20 ms before each m.e.p.s.c. **Bb**, Average time course of changes in m.e.p.s.c. amplitudes normalized to the mean Pre value in each experiment (average = 10.0 pA for 8-Br-cGMP and 12.6 pA for control, not significantly different). Each point represents the average during a 1 to 4-min period. **C**, No enhancement of m.e.p.s.c. frequency or amplitude by postsynaptic injection of cGMP. **Ca**, Average time course of changes in m.e.p.s.c. frequency normalized to the Pre value in each experiment (average Pre = 114.8  $\text{min}^{-1}$ ). Each point represents the average during a 1 min period. **Cb**, Average time course of changes in m.e.p.s.c. amplitude normalized to Pre (average Pre = 10.7 pA).

**METHODS.** The bath solution contained 1  $\mu$ M TTX, which completely

blocked spontaneous action potentials. Ruptured whole-cell patch recording was used. The electrode solution contained (in mM): caesium gluconate (110); KCl (10); HEPES (5); EGTA (0.6);  $\text{MgCl}_2$  (5); ATP (2); pH 7.1; 310 mosM, adjusted with sucrose. For intracellular injection of cGMP, the tip of the electrode was first filled with normal electrode solution and then the rest of the electrode was back-filled with the same solution containing 50  $\mu$ M cGMP. The diffusion time for cGMP to reach the distal processes was estimated to be less than 5 min in other experiments in which movement of a similar-sized molecule (Lucifer Yellow) was observed under a fluorescence microscope. A continuous record of the experiments was stored on an analogue tape recorder and then selected portions were digitized and analysed with an Axobasic program written by A. Kyzioz. Events were included in the analysis if they exceeded a preset threshold above the noise level (usually between 2 and 3 pA) and had a rapid rise-time and an acceptable waveform. We believe that we were able to record most of the m.e.p.s.cs because (1) the distribution of m.e.p.s.c. amplitudes had little overlap with the noise distribution (**Ba**), and (2) changing the postsynaptic holding potential from -60 mV to -90 mV (and thus increasing the amplitude of the m.e.p.s.cs by increasing the driving force) did not produce any increase in m.e.p.s.c. frequency ( $n = 3$ ,  $\bar{x} = 2.6\%$  decrease, not significant). Events summing with previous events were included in the frequency analysis but were not included in the amplitude analysis. Only experiments with at least 25 m.e.p.s.cs per min during the Pre period were included in the amplitude analysis. M.e.p.s.c. amplitudes were measured between the peak of the event and the mean of a 2-ms baseline period 20 ms before the start of the event. Noise amplitudes were measured at the starting point of each baseline period.

not with weak tetanic stimulation of the presynaptic neuron. All experiments were conducted with normal  $Mg^{2+}$  and 50  $\mu M$  APV in the bath. Presynaptic injection of cGMP (50  $\mu M$ ) paired with tetanus produced an immediate enhancement of the e.p.s.c. that lasted for the remainder of the experiment (Fig. 3A). Presynaptic injection of cGMP alone produced no significant effect in these experiments, nor did presynaptic injection of either the vehicle ( $n=3$ ) or cAMP (50  $\mu M$ ,  $n=3$ ) paired with tetanus. Somewhat surprisingly, postsynaptic injection of cGMP paired with tetanus produced a long-lasting decrease in the e.p.s.c. that was significant compared with the pre-training level, but was not significant compared with the control experiments.

These results demonstrate that a presynaptic increase in cGMP can induce activity-dependent long-lasting enhancement of the e.p.s.c. To attempt to determine whether the expression of that enhancement is presynaptic or postsynaptic, we first examined the standard deviation divided by the mean or the coefficient of variation (c.v.) of e.p.s.c. amplitudes. At the neuromuscular junction, a change in c.v. indicates a presynaptic change in transmitter release and c.v.<sup>-2</sup> is an estimate of the number of quanta released<sup>25</sup>, although changes in c.v. at central synapses might also involve postsynaptic modifications<sup>26</sup>. LTP in slices is accompanied by an increase in c.v.<sup>-2</sup> proportional to the increase in e.p.s.c. amplitude<sup>15,27</sup>. Similarly, potentiation by presynaptic injection of cGMP paired with weak tetanic stimulation was accompanied by an increase in c.v.<sup>-2</sup> that paralleled the increase in e.p.s.c. amplitude (Fig. 3Ba). There was an increase in c.v.<sup>-2</sup> after all training procedures in Figs 2Bb, 3Ab and 3Ba that produced potentiation of the e.p.s.c. (Fig. 3Bb). This increase correlated significantly with the increase in mean e.p.s.c. amplitude ( $r=0.89$ ,  $t=5.11$ ,  $P<0.01$ ), and the slope of the regression line was close to one ( $1.11\pm0.22$ ).

As a second way of distinguishing between presynaptic and postsynaptic effects of cGMP, we examined spontaneous miniature synaptic currents (m.e.p.s.c.s). Bath application of 8-Br-cGMP (50 or 100  $\mu M$ ) for about 1 min produced an immediate enhancement of the frequency of spontaneous m.e.p.s.c.s that lasted for the remainder of the experiment (Fig. 4A). In three experiments that lasted 2 h the m.e.p.s.c. frequency was still enhanced at that time ( $\bar{x}=181\%$  increase). In control experiments with normal saline perfusion there was no consistent change in m.e.p.s.c. frequency for up to 90 min. These results suggest that potentiation by 8-Br-cGMP is accompanied by a change in presynaptic transmitter release independent of changes in ionic currents during the depolarizing step. In contrast, we did not observe any increase in the amplitudes of the spontaneous m.e.p.s.c.s (Fig. 4B), suggesting that the potentiation does not depend on a postsynaptic change in receptor sensitivity, in agreement with the results of the c.v. analysis. Unlike perfusion with 8-Br-cGMP, intracellular injection of cGMP into the postsynaptic cell did not produce an increase in m.e.p.s.c. frequency (Fig. 4C). This result implies that, like potentiation of the evoked e.p.s.c., the increase in m.e.p.s.c. frequency by 8-Br-cGMP (Fig. 4A) results from a direct action in the presynaptic neurons.

We have found that it is possible to produce reliable long-lasting potentiation in culture with properties similar to LTP in slices, and have taken advantage of the culture system to investigate the role of cGMP. Our results are consistent with the results of previous experiments that used field recording in hippocampal slices<sup>12</sup> and in addition provide new evidence supporting a presynaptic function of cGMP. A c.v. analysis and an analysis of spontaneous m.e.p.s.c.s suggest that potentiation by cGMP involves presynaptic enhancement of transmitter release. These results are similar to results that have been obtained for potentiation by tetanic stimulation, glutamate or nitric oxide<sup>15,17</sup>, suggesting that cGMP-induced potentiation may share similar mechanisms. In addition, we find that potentiation can be produced by injection of cGMP into the presynaptic, but not the postsynaptic, neuron, providing a new type of evidence that LTP involves presynaptic events. Because potentiation normally also

requires postsynaptic events (Fig. 1C, D), these results support the idea that cGMP may be the presynaptic effector of a retrograde messenger. □

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## Coupling of bitter receptor to phosphodiesterase through transducin in taste receptor cells

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THE rod and cone transducins are specific G proteins originally thought to be present only in photoreceptor cells of the vertebrate retina<sup>1–4</sup>. Transducins convert light stimulation of photoreceptor opsins into activation of cyclic GMP phosphodiesterase (reviewed in refs. 5–7). A transducin-like G protein, gustducin, has been identified and cloned from rat taste cells<sup>8</sup>. We report here that rod transducin is also present in vertebrate taste cells, where it specifically activates a phosphodiesterase isolated from taste tissue. Furthermore, the bitter compound denatonium in the presence of taste-cell membranes activates transducin but not  $G_i$ . A peptide that competitively inhibits rhodopsin activation of

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transducin<sup>9</sup> also blocks taste-cell membrane activation of transducin, arguing for the involvement of a seven-transmembrane-helix G-protein-coupled receptor. These results suggest that rod transducin transduces bitter taste by coupling taste receptor(s) to taste-cell phosphodiesterase. Phosphodiesterase-mediated degradation of cyclic nucleotides may lead to taste-cell depolarization through the recently identified cyclic-nucleotide-suppressible conductance<sup>10</sup>.

Using the polymerase chain reaction (PCR), we isolated clones for the  $\alpha$ -subunits of rod and cone transducin from a taste cell complementary DNA library. The entire coding sequence of rat rod transducin ( $\alpha_{t-rod}$ ) was obtained by cloning both the retinal and taste cDNAs. The DNA sequence of taste-cell-derived  $\alpha_{t-rod}$  was identical to the sequence of retinal  $\alpha_{t-rod}$ , except that the taste cDNA contained an additional ~250 base pairs (bp) of 5' untranslated sequence (data not shown). At the amino-acid level, the inferred rat  $\alpha_{t-rod}$  protein is 99.7% identical to mouse  $\alpha_{t-rod}$  and 79% identical to rat  $\alpha$ -gustducin.

RNase protection assays (Fig. 1a) confirmed that  $\alpha_{t-rod}$  messenger RNA was present in taste tissue and retina, but absent from non-taste lingual tissue, olfactory epithelium, brain, liver, heart or kidney (consistent with previous northern-blot data<sup>2</sup>). There is several hundred-fold more  $\alpha_{t-rod}$  mRNA in retina than in taste tissue (Fig. 1a), and ~25-fold more gustducin mRNA than  $\alpha_{t-rod}$  mRNA in taste tissue (data not shown). RNase protection assays did not detect  $\alpha_{t-cone}$  mRNA in taste tissue (data not shown): presumably  $\alpha_{t-cone}$  mRNA is expressed at very low levels in taste tissue.

Immunoprecipitation of ADP-ribosylated G-protein  $\alpha$ -subunits demonstrated the presence of  $\alpha_{t-rod}$  protein in taste-bud-containing tissue, but not in the non-sensory lingual epithelium (Fig. 1b). Preincubation of the antiserum with rod transducin peptide blocked immunoprecipitation of transducin (Fig. 1b). Furthermore, western blot analysis with a rod-transducin-specific antiserum also demonstrated the presence of  $\alpha_{t-rod}$  pro-

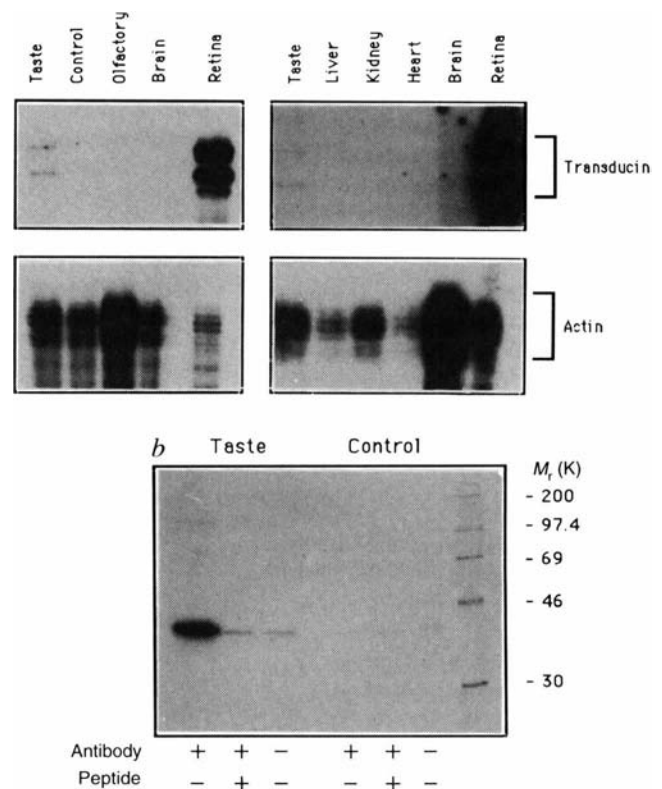
tein in taste tissue and retina, and its absence from non-sensory lingual tissue (data not shown).

To determine directly whether  $\alpha_{t-rod}$  mRNA and protein are present within the taste buds, *in situ* hybridization (data not shown) and immunohistochemistry (Fig. 2) were done on taste-bud sections. Rod transducin protein was found in the receptor cells of the taste buds of the circumvallate papilla (Fig. 2a) and the photoreceptor cells of the retina (data not shown), but not elsewhere in the non-sensory portions of the taste tissue. Preincubation of the antiserum with rod transducin peptide blocked immunoreactivity in retina (data not shown) and taste buds (Fig. 2b); preincubation of the transducin antiserum with the corresponding gustducin peptide had no effect (Fig. 2c) (that is, the immunoreactivity is transducin-specific). Similar studies using a gustducin-specific antiserum demonstrated the presence of gustducin in the taste buds of the circumvallate papilla (Fig. 2d, e, f). These antisera specifically distinguish between gustducin and  $\alpha_{t-rod}$ , and demonstrate that both proteins are present in taste buds. In the absence of primary antibody no immunoreactivity is detected (Fig. 2g).

To determine whether  $\alpha_{t-rod}$  expression is dependent on the presence of taste buds, immunohistochemistry was carried out with frozen sections taken from rats whose tongues had been denervated. Unilateral sectioning of the glossopharyngeal and chorda tympani nerves causes the taste buds of the ipsilateral foliate papilla to degenerate, but leaves the taste buds of the contralateral foliate papilla intact<sup>11</sup>. After unilateral sectioning of the left chorda tympani and left glossopharyngeal nerve, the left foliate papilla was devoid of taste buds and displayed no detectable rod transducin (Fig. 2h) or gustducin (Fig. 2j). However, the contralateral (right) foliate papilla retained taste buds that express rod transducin (Fig. 2i) and gustducin (Fig. 2k).

To determine whether rod transducin interacts with a phosphodiesterase (PDE) in taste tissue, we isolated PDEs from

FIG. 1 Expression of  $\alpha_{t-rod}$  in rat taste tissue. a, Left, expression of  $\alpha_{t-rod}$  (top) and actin (lower) transcripts assayed by RNase protection of *in vitro* generated RNA.  $\alpha_{t-rod}$  transcripts are present in the taste-cell-enriched library (taste), and the retinal library;  $\alpha_{t-rod}$  RNA is absent from non-taste lingual epithelium (control), brain and olfactory epithelium. The retina sample contained 0.4  $\mu$ g RNA, other samples 2  $\mu$ g RNA. Right, RNase protection with total RNA (15  $\mu$ g) demonstrates that  $\alpha_{t-rod}$  is absent from liver, kidney, heart and brain but present in retina and taste tissue. b, Expression of  $\alpha_{t-rod}$  protein in bovine taste tissue. Membrane extracts were radioactively labelled by pertussis-toxin-mediated ADP ribosylation then transducin protein immunoprecipitated by an  $\alpha_{t-rod}$ -specific antiserum (Ab<sup>+</sup> lanes). Preincubation of the antiserum with the rod transducin peptide (peptide<sup>+</sup> lanes) blocks the immunoprecipitation (that is, the immunoprecipitate in taste tissue is  $\alpha_{t-rod}$ ). In the absence of transducin antiserum (Ab<sup>-</sup> lanes), some nonspecific trapping of ADP-ribosylated  $\alpha$ -gustducin (Taste) and  $\alpha$  (Taste and Control) occurs. METHODS. PCR, RNA purification, *in vitro* RNA generation, <sup>32</sup>P-labelled probes, PCR primers, actin clone and RNase protection assay have been described<sup>8</sup>. The template for  $\alpha_{t-rod}$  probes corresponds to the region encoding amino acids 206 to 268; the  $\alpha_{t-cone}$  template encodes amino acids 256 to 341. For immunoprecipitation, bovine circumvallate papillae were dissected away from non-taste tissue, chilled on ice in 25 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, PMSF (100  $\mu$ g ml<sup>-1</sup>), homogenized with a Dounce homogenizer, and the lysate cleared (10,000 r.p.m. for 5 min). Then nicotinamide adenine [adenylate-<sup>32</sup>P] dinucleotide (NAD; Amersham) and pertussis toxin (Sigma) were added<sup>28</sup>. <sup>32</sup>P-ADP-ribosylated transducin was immunoprecipitated by anti-transducin-specific antiserum and protein A beads (Bio-Rad). The rabbit polyclonal antiserum was raised against a rod transducin-specific peptide (amino acids 85 to 103) (from M. Lochrie, J. Watson and M. Simon). Control experiments were with non-taste lingual tissue. Immunoprecipitated samples were boiled in 2% SDS and 100 mM DTT, separated from the protein-A beads by centrifugation, then electrophoresed through SDS-polyacrylamide gels. To ensure the specificity of immunoprecipitated products, control experiments were performed in which 150  $\mu$ M rod transducin peptide was preadsorbed to the transducin antiserum before immunoprecipitation.





bovine taste and non-taste lingual tissues, then assayed for stimulation by the activated  $\alpha$ -subunit of transducin or by a transducin-derived peptide that mimics the effects of the activated G proteins<sup>12</sup>. The effector interaction region of  $\alpha_{t-rod}$  differs from that of  $\alpha$ -gustducin at seven amino acids. Furthermore, the  $\alpha$ -gustducin-derived peptide is incapable of activating rod

PDE<sup>13</sup>. If the analogous effector-interaction region of gustducin is involved in its presumed activation of taste PDE, then the gustducin and transducin peptides may be used to assay for taste PDE activation.

Two chromatographically distinct peaks of PDE activity were identified in both taste (Fig. 3a) and non-taste (Fig. 3b) tissues.

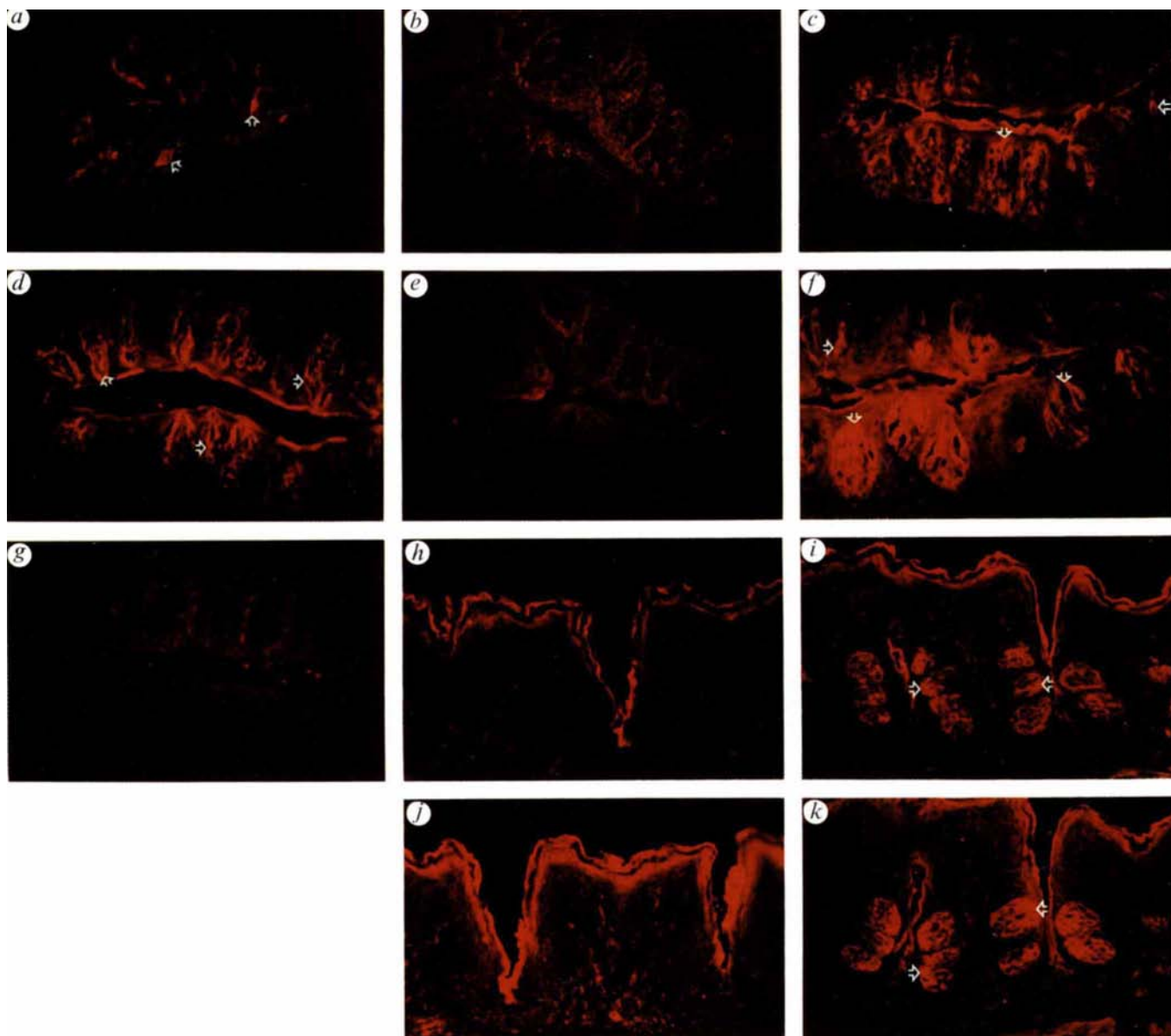


FIG. 2 Immunofluorescence photomicrographs of rat taste papillae treated with  $\alpha$ -gustducin or  $\alpha_{t-rod}$  specific antibodies and rhodamine-conjugated secondary antibody. *a*, Circumvallate papilla reacted with anti-transducin antiserum; *b*, circumvallate papilla, anti-transducin antiserum pre-blocked by rod transducin peptide; *c*, circumvallate papilla, anti-transducin antibody adsorbed to gustducin peptide (this peptide does not block transducin immunoreactivity); *d*, circumvallate papilla, anti-gustducin antibody; *e*, circumvallate papillae, anti-gustducin antibody pre-blocked by gustducin peptide; *f*, circumvallate papilla, anti-gustducin antibody adsorbed to rod transducin peptide (this peptide does not block gustducin immunoreactivity); *g*, circumvallate papilla, no primary antibody; *h*, ipsilateral (left) foliate papilla from a rat denervated for the left glossopharyngeal and left chorda tympani nerves, reacted with anti-transducin antiserum; *i*, contralateral (right) foliate papilla, anti-transducin antiserum; *j*, ipsilateral (left) foliate papilla, anti-gustducin antiserum; *k*, contralateral (right) foliate papilla,

anti-gustducin antiserum. Open arrows indicate taste receptor cells expressing rod transducin (*a*, *c*, *i*) or gustducin (*g*, *f*, *k*). Original magnification was  $400\times$  (*a*–*g*) or  $300\times$  (*h*–*k*).

**METHODS.** Tongues were fixed in 4% paraformaldehyde in PBS, transferred to 20% sucrose in PBS, then stored at  $4^{\circ}\text{C}$ . Ten-micron-thick frozen sections were rinsed with PBS, then blocked for 30 min to 1 h in blocking solution (0.3% Triton, 1% goat or horse serum in PBS). After removal of blocking solution, gustducin antibody (at 1:100) or rod transducin antibody (at 1:20) (in 0.3% Triton, 2% BSA in PBS) was added. Preincubation of antibody with gustducin or rod transducin peptide (50  $\mu\text{M}$  final concentration) was for 30 min to 1 h on ice in 0.3% Triton, 2% BSA in PBS. A gustducin-specific peptide (amino acids 95–109) synthesized on a branched lysine core was used to inoculate rabbits; by the fourth monthly booster injection, the antiserum titre was  $\sim 1:500$ . Unilateral nerve sectioning was done as described<sup>8</sup>.

Peak I from taste tissue showed a fourfold increase in cGMP PDE activity upon the addition of the rod transducin peptide (peptide 1) (Fig. 3c); peak I from non-taste tissue was not stimulated by the rod transducin peptide. The gustducin effector interaction peptide (peptide 3) did not affect either taste or non-taste peak I activities (Fig. 3c). Addition of the rod peptide to peak II from either taste or non-taste tissues led to a decrease in cGMP PDE activity compared to basal levels (data not shown). Activated rod transducin protein stimulated taste peak I cGMP PDE activity more than tenfold, whereas non-taste peak I activity was unaffected by transducin protein (Fig. 3d). In sum, we have identified a PDE in taste tissue that is stimulated by rod transducin or the rod transducin effector interaction peptide but not by the gustducin peptide. Presumably this PDE is the taste-cell target of transducin action during taste transduction. However, it is premature to conclude that gustducin does not activate this same PDE, because gustducin protein may interact with PDE other than through the effector-interaction region or the gustducin-derived peptide may not mimic the action of activated gustducin protein.

To determine whether taste-cell transducin couples to a taste receptor, we isolated membranes from bovine tongue tissues, reconstituted them with transducin, then assayed for transducin activation. We used trypsin sensitivity<sup>14,15</sup> and GTP- $\gamma$ S binding<sup>16</sup> to monitor activation of transducin by rhodopsin (positive control), taste membranes or non-taste lingual membranes in the presence of various tastants (Fig. 4). In the absence of membranes, in the presence of non-taste membranes, or in the presence of taste membranes without added tastants, there was no activation of transducin (evidenced by the fragment of  $M_r$  23K derived from GDP-bound transducin) (Fig. 4a). Three different tastants (quinine, saccharin and denatonium) were assayed for their ability to activate transducin. Only bitter denatonium activated transducin (evidenced by the 32K fragment derived from GTP $\gamma$ S-bound transducin) and only in the presence of taste membranes (Fig. 4a): quinine and saccharin did not activate transducin, and denatonium in the absence of taste membranes likewise did not activate transducin. In the presence

of taste membranes, activation of transducin could readily be detected with 100  $\mu$ M denatonium (Fig. 4a, b). But in the presence of non-taste membranes, even 10 mM denatonium failed to activate transducin (Fig. 4a, b). Presumably a denatonium-responsive receptor present in the taste-membrane extract couples to the exogenously added transducin. Taste aversion in mice requires 50–100  $\mu$ M denatonium (G. Wong and R.F.M., unpublished results) whereas 10  $\mu$ M denatonium leads to inositol trisphosphate ( $\text{InsP}_3$ ) generation in rat taste cells<sup>17</sup>; it is not known what concentration of denatonium is required to activate bovine taste cells or lead to bovine aversion.

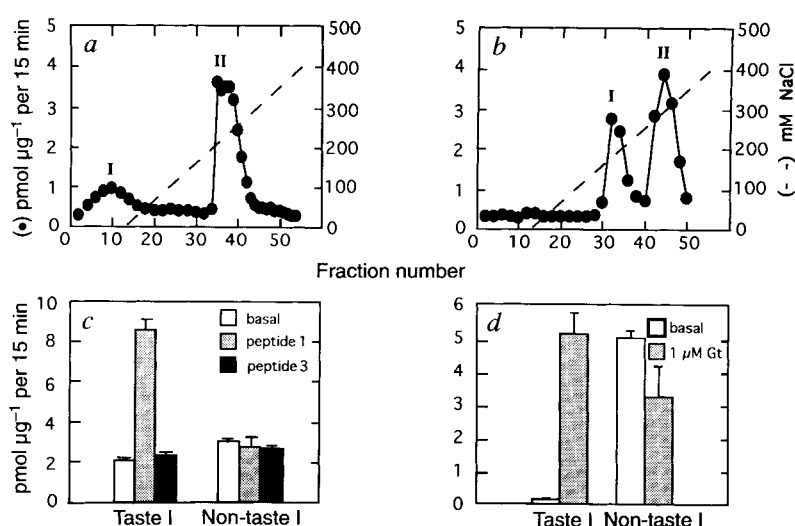
Neither rhodopsin nor taste membranes plus denatonium activated  $G_i$  (assayed by GTP- $\gamma$ S binding), whereas denatonium in the presence of taste membranes or activated rhodopsin alone activated transducin ( $\sim 4$ -fold and  $\sim 10$ -fold, respectively; Fig. 4c). This argues for a taste-specific receptor that couples to transducin but not to the similar G protein  $G_i$ . Preliminary experiments suggest that this receptor also couples to and activates gustducin (L.R.-A. and R.F.M., unpublished results).

A peptide derived from the C-terminal region of transducin (amino acids 311–329) competitively inhibits the rhodopsin-transducin interaction<sup>9</sup>. This peptide blocked rhodopsin-mediated activation of transducin (data not shown) and likewise blocked taste-receptor activation of transducin, whereas a control peptide derived from  $G_i$  did not interfere with transducin activation (Fig. 4d). These results suggest that a seven-transmembrane-helix taste receptor binds denatonium and activates transducin by interaction with its carboxy terminus. However, denatonium does not nonspecifically enhance the rhodopsin-transducin interaction (data not shown).

The direct demonstration that rod transducin is present in taste cells where it activates a taste-tissue PDE and is in turn activated by a denatonium-responsive taste receptor, provides insight into the possible role(s) of transducin in taste transduction. Bitter compounds have been correlated with PDE activation<sup>18</sup> and several PDE inhibitors enhance sweet perception<sup>19,20</sup>. We have cloned portions of two different cAMP (type IV) PDEs from rat taste tissue<sup>21</sup> but do not yet know

FIG. 3 Phosphodiesterase (PDE) isolated from bovine taste tissue is activated by rod transducin. Fractionation of PDE activity from a, bovine circumvallate tissue, and b, non-taste lingual tissue reveals two peaks of PDE activity in each tissue; c, assays of taste versus non-taste peak I cGMP PDE activity: the rod transducin effector interaction peptide (grey bars, peptide 1) activates taste peak I but not non-taste peak I; the gustducin peptide (black bars, peptide 3) does not stimulate either taste or non-taste peak I; basal activity (open bars) in the absence of added peptide; d, assays of taste versus non-taste peak I cGMP PDE activity: activated  $\alpha$ -rod transducin protein (grey bars, 1  $\mu$ M  $G_t$ ) activates taste peak I but not non-taste peak I; basal activity (open bars) in the absence of activated  $\alpha$ -rod.

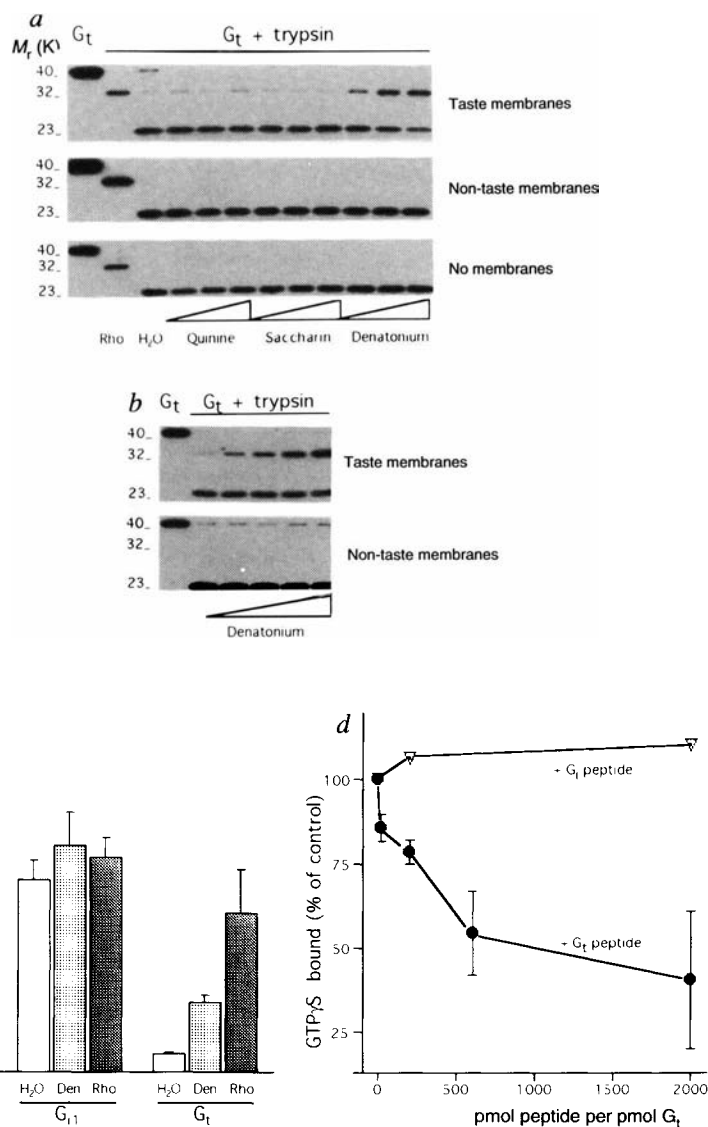
**METHODS.** Bovine circumvallate papillae and non-gustatory lingual epithelia were collected and rapidly frozen in liquid nitrogen; subsequent purification procedures were carried out at 4 °C. Tissues were homogenized in DEAE buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 10  $\mu$ g  $\text{ml}^{-1}$  pepstatin A, 10  $\mu$ g  $\text{ml}^{-1}$  leupeptin, 10  $\mu$ g  $\text{ml}^{-1}$  aprotinin, and 100  $\mu$ M AEBF (4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride)) with a rotorstator homogenizer. Centrifugation removed particulate (1,500g) and membrane fractions (45,000g). The supernatant was applied to a DEAE-sephacel column, the column washed with DEAE buffer, and PDEs eluted with a 0–600 mM NaCl linear gradient in DEAE buffer. PDE activity was measured as described<sup>29</sup>. Peak column fractions were pooled, concentrated, snap-frozen in dry-ice-methanol, and stored at  $-80$  °C. Protein values were determined using the Bio-Rad protein assay reagent. Effector peptides were used at a final concentration of 200  $\mu$ M. Results are the average  $\pm$  s.e.m. of triplicate determinations and are



representative of three independent tissue preparations. 22-amino-acid-long effector-interaction peptides corresponding to amino acids 293–315 of  $\alpha$ -rod or amino acids 297–318 of  $\alpha$ -gustducin were synthesized and purified as described<sup>13</sup>. Rod transducin was purified as described<sup>30</sup> with an additional gel-filtration chromatography step through AcA 44. Aluminium-magnesium fluoride (AMF) activation of transducin was in a reaction mix of 5 mM NaF and 50  $\mu$ M  $\text{AlCl}_3$ ; the PDE assay buffer was 10 mM in  $\text{MgCl}_2$ .

FIG. 4 Reconstituted bovine taste receptor activates transducin. **a**, Tryptic patterns distinguish GTP $\gamma$ S-transducin ( $M_r$  32K) from GDP-bound transducin (23K fragment) from undigested transducin ( $\sim$ 40K). Transducin was mixed with taste membranes (top), non-taste membranes (middle) or no membranes (bottom). In the presence of trypsin, rhodopsin (Rho, positive control) generates a 32K fragment, water (negative control) generates a 23K fragment. The effect of various tastants on transducin activation was assessed. Neither quinine (2.5  $\mu$ M, 25  $\mu$ M, 250  $\mu$ M) nor saccharin (100  $\mu$ M, 1 mM, 10 mM) activated transducin, whereas denatonium (100  $\mu$ M, 1 mM, 10 mM) activated transducin (note the shift from 23K (GDP-bound) to 32K (GTP $\gamma$ S bound)). **b**, Dose response of denatonium (0, 100  $\mu$ M, 300  $\mu$ M, 900  $\mu$ M, 3 mM)-mediated activation of transducin: taste membranes (top) versus non-taste membranes (lower). **c**, Taste-receptor activates transducin but not  $G_i$ . Samples contained taste membranes and  $G_i$  or transducin; water, denatonium (Den) or rhodopsin (Rho) were added as indicated. GTP- $\gamma$ S binding to  $G_i$  is not stimulated by either denatonium or rhodopsin. **d**, Competitive inhibition of taste-receptor-mediated activation of transducin by a transducin peptide but not by a  $G_i$  peptide.

**METHODS.** Bovine taste and non-taste membranes were prepared as described for Fig. 3. Frozen tissue (0.5–2.0 g) was homogenized in DEAE buffer (10 ml), then the 45,000g pellets were washed twice with DEAE buffer lacking protease inhibitors. Membrane fractions were resuspended in DEAE buffer (250–500  $\mu$ l) lacking protease inhibitors, then further homogenized by 10 passages through 25-gauge needles. G-protein activation by taste receptors was as follows: taste or non-taste membranes were resuspended at 0.25 mg ml $^{-1}$  in incubation buffer (25 mM Tris, pH 7.5; 1 mM MgCl $_2$ ; 150 mM NaCl; 5 mM DTT; 1 mM CHAPS; 100  $\mu$ M GDP; 1  $\mu$ M GTP- $\gamma$ S). For experiments without added membranes, BSA (0.25 mg ml $^{-1}$ ) in incubation buffer was added. The diluted samples were mixed on ice with purified rod transducin or recombinant  $G_i$  (0.3–0.6  $\mu$ M final concentration). Quinine sulphate, sodium saccharin and denatonium benzoate were all from Sigma. Rhodopsin was used at 0.1–1  $\mu$ M final concentration. Mixtures were incubated for 0.5–1 h at 30  $^{\circ}$ C, then analysed by trypsin digestion<sup>14,15</sup> or by GTP- $\gamma$ S binding<sup>16</sup>. For trypsinizations, TPCK-treated trypsin (Sigma) was added (1:25 trypsin to total protein) after the incubation: digestions were at room temperature (1–1.5 h) and were stopped by adding soybean trypsin inhibitor. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Intact  $\alpha_t$ -rod and its 23K and 32K tryptic fragments were visualized using transducin-specific antibody and the ECL system (Amersham). GTP- $\gamma$ S binding was halted by diluting the samples 1:50 in ice-cold washing buffer (20 mM Tris, pH 8.0, 100 mM NaCl and 25 mM MgCl $_2$ ) then rapidly filtering through 0.45- $\mu$ m nitrocellulose membranes (Millipore; type HA). Filters were washed (3 $\times$ ) with cold washing buffer (5 ml), air-dried and counted. Basal values without added G protein were  $\leq$ 5% of the maximum value obtained upon activation. All binding experiments were done in duplicate, results are aver-



whether either of these cloned PDEs corresponds to the PDE that we have partially purified from bovine taste tissues. Preliminary pharmacological characterization of the bovine taste PDE suggests that it is distinct from the retinal cGMP PDEs (D.W. and R.F.M., unpublished). Our reconstitution of a denatonium-responsive receptor from bovine taste tissue is, to our knowledge, the first direct demonstration of a role for a seven-transmembrane-helix G-protein-coupled receptor in taste transduction. PCR has been used to clone G-protein-coupled receptors from taste tissue<sup>22–24</sup>, but the expression patterns of these receptors suggest that they are olfactory receptors that are not expressed in taste tissues or taste cells. Our data indicate that transducin is involved in bitter transduction via a cascade similar to that in vision; that is, bitter receptor(s) activate taste-cell transducin, which in turn activates PDE to decrease the levels of intracellular 3', 5'-cyclic nucleotides. Decreased levels of cyclic

nucleotides would be antagonistic to the sweet transduction pathway (sweet compounds apparently activate  $G_s$ , and thereby adenyl cyclase, to generate cAMP<sup>25–27</sup>), suggesting that transducin may also be involved in the antagonism of sweet transduction. In the accompanying paper<sup>10</sup> we describe a taste-cell conductance that leads to cation entry when cyclic nucleotide levels are decreased; bitter receptor activation of transducin (and thereby PDE) would decrease taste-cell cyclic nucleotide levels, leading to taste-cell depolarization via this conductance. □

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## A cyclic-nucleotide-suppressible conductance activated by transducin in taste cells

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TASTE can be divided into four primary sensations: salty, sour, sweet and bitter. Salty and sour are directly transduced by apical channels<sup>1–4</sup>, whereas sweet and bitter utilize cyclic nucleotide second messengers<sup>5–11</sup>. We have shown that rod transducin is present in mammalian taste receptor cells, where it is activated by a bitter receptor and in turn activates a phosphodiesterase<sup>12</sup>. Here we introduce into frog taste cells peptides derived from transducin's phosphodiesterase-interaction region, which cause an inward whole-cell current in a subset of cells. We find that the peptides' effects are reversibly suppressed by IBMX and forskolin, indicative of a transducin-activated phosphodiesterase. Cyclic nucleotides suppress the whole-cell current, indicating that cyclic nucleotides may regulate taste-cell conductance. IBMX modifies taste-cell responses to two taste stimuli, implicating phosphodiesterase in taste transduction. Submicromolar cyclic nucleotides directly suppress the conductance of inside-out patches derived from the taste-cell plasma membrane, independently of protein phosphorylation. The channels are unusual in that they are suppressed, rather than activated by cyclic nucleotides. We propose that transducin, via phosphodiesterase, decreases cyclic nucleotide levels to activate the cyclic-nucleotide-suppressible conductance, leading to Ca<sup>2+</sup> influx and taste-cell depolarization.

Gustducin is a G protein specific to taste receptor cells (TRCs) which is closely related to the transducins<sup>13</sup>. Introduction into TRC of gustducin, transducin or their effector-interaction peptides provides a means to assay the roles of G proteins, phosphodiesterase (PDE) and cyclic nucleotide(cNMP)-regulated channels in taste transduction. Three transducin-derived peptides (peptide 1 ( $\alpha_{t-rod}$  amino acids 293–314), peptide 25 ( $\alpha_{t-rod}$  amino acids 293–307) and peptide 66 ( $\alpha_{t-rod}$  amino acids 311–329)) were introduced by patch pipette into bullfrog (*Rana catesbeiana*) TRCs. Peptides 1 and 25 are derived from transducin's PDE interaction region and fully activate bovine (refs 14 and 15, and N. Spickofsky and R.F.M., unpublished results) and frog S. I. Zharikov, A. V. Kosolapov and S.S.K., unpublished results) rod PDEs, and a taste-tissue-specific PDE<sup>12</sup>. Peptide 66 is a control peptide derived from transducin's rhodopsin inter-

action region that doesn't activate PDE<sup>12,14</sup>. When patch pipettes contained only 1 mM ATP (>100 cells) or ATP and 100  $\mu$ M peptide 66 (28 cells) TRC whole-cell (WC) currents generally decreased over 5–10 min (subsequent to establishment of the whole-cell mode) and then remained relatively stable (for 20–100 min) (Fig. 1c, d). A small fraction (7–10%) of TRC gave short-lived (5–15 min) WC recordings accompanied by unstable or increased WC currents which were not suppressed by the PDE inhibitor IBMX (200  $\mu$ M).

The superfusion of TRC with peptides 1 and 25 induced an inward WC-current elevation (~2–10-fold over baseline) and an increase in TRC membrane conductance (Fig. 1f) ~1–3 min after disruption of the patch membrane (Fig. 1a). Extracellular application of IBMX (100–200  $\mu$ M) (Fig. 1a) or the adenylyl cyclase activator forskolin (0.5  $\mu$ M) (data not shown) reversibly suppressed (from 20 to 100%) the WC-current increase, consistent with PDE activation being responsible for the peptide-induced current increase. Thirteen of 83 cells (15%) displayed an IBMX-blockable current in response to peptide 1; a similar response to peptide 25 was found in 4 of 25 cells (16%). Eighty of the other 91 cells displayed a relatively steady WC current; 11 of the 91 cells showed unstable increased WC currents that were not suppressed by IBMX.

When patch pipettes contained saline without ATP, the TRC WC currents rapidly become unstable and tended to increase (17 cells); these currents were never suppressed by IBMX. Introduction of cNMP (generally 8-bromo-derivatives to avoid degradation by PDE) by the patch pipette dramatically changed WC-current behaviour in some cells. Suppression of inward WC currents (from 50 to 90%) was accompanied by a reduction in TRC membrane conductance (Fig. 1g) observed upon the introduction of 20  $\mu$ M 8-Br-cGMP, 20  $\mu$ M 8-Br-cAMP or 10  $\mu$ M 8-BrGMP + 10  $\mu$ M 8-Br-cAMP (8 of 20, 2 of 7 and 2 of 5 cases, respectively). In some experiments, perfused patch pipettes<sup>16</sup> were used to regulate the initiation of cNMP application and provide control recordings. In certain of these cases the WC current was initially stable, then ~20–50 seconds after commencing patch pipette perfusion, the inward current rapidly dropped (for example, with 8-Br-cGMP; Fig. 1b). Taken together these data suggest that ~15% of TRC express the transducin-regulated PDE and that cNMPs directly or indirectly inhibit the TRC plasma membrane conductance.

To determine whether the transducin/PDE regulated conductance is involved in taste transduction, we examined TRC responses to some tastants. Bath application of 5 mM saccharin evoked a slow response with ~15 pA amplitude (Fig. 1c). Simultaneous bath application of saccharin and IBMX also evoked a decreased amplitude response, but with apparently slowed kinetics: the TRC is still responsive, as 10 mM saccharin alone induced a relatively fast, 20 pA response. The high-potency artificial sweetener NC-01 (NC-00274-01; ref. 10) evoked a qualitatively different response (Fig. 1d). Simultaneous application of NC-01 and IBMX produced a smaller response with a

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prolonged duration; continuous application of 0.4 mM IBMX suppressed the TRC responsiveness to NC-01 (Fig. 1d). The  $\text{Na}^+$  channel blocker amiloride did not suppress inward WC currents recorded from TRC that were sensitive to saccharin and NC-01; moreover amiloride and saccharin evoked similar responses (Fig. 1e, d). These observations indicate that amiloride-blockable  $\text{Na}^+$  channels do not participate in NC-01 or saccharin transduction of frog TRC. Results from hamster TRC also indicate that the amiloride-blockable  $\text{Na}^+$  conductance is not involved in sweet transduction<sup>10</sup>.

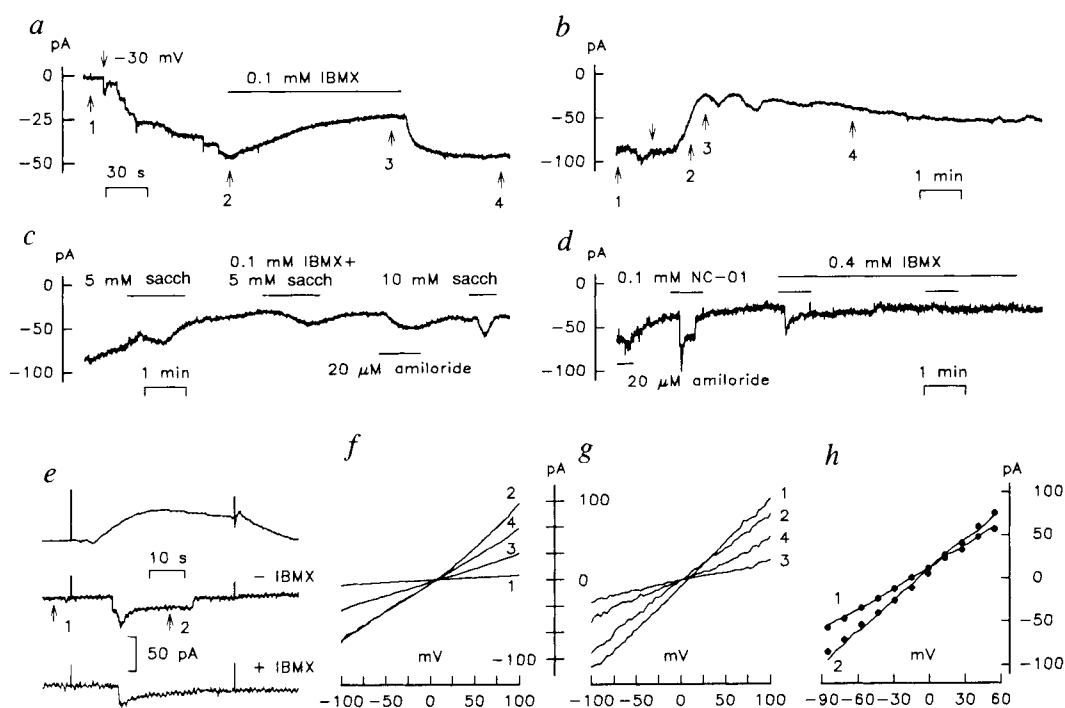
Very few TRC responded to a given taste stimulus: 3 of 45 cells (6.6%) responded to 5 mM saccharin; 4 of 57 cells (7%) responded to 0.1 mM NC-01; only 1 cell of 23 (4%) responded to 0.1 mM denatonium (bitter to humans). This cell also responded to NC-01 (Fig. 1e, h). NC-01 evoked biphasic responses that appeared to be the superposition of step-like and transient currents (Fig. 1e), implying multiple ion transporting and/or transduction processes. Without IBMX (4 cells) the response latency to NC-01 was about 4–6 seconds; with IBMX (2 cells), the delay increased to 8–10 seconds, the step-like phase was sup-

pressed and the transient phase prolonged (the IBMX effects were reversible; data not shown). Thus, when applied at the same time as taste stimuli, the membrane-permeant PDE-inhibitor IBMX can modify the very first phases of TRC responses. This suggests a direct action of IBMX on the transduction machinery, implying PDE participation in saccharin and NC-01 transduction.

Taste stimuli generally evoke depolarization<sup>1,4</sup>; neither basolateral  $\text{K}^+$  channels<sup>11</sup> nor the previously known cNMP-gated cation channels<sup>17,21</sup> can serve as the terminal element of a taste pathway based upon PDE activation (that is, cNMP decrease). To identify the ion channels underlying the cNMP-dependent conductance, we recorded from excised patches. When both sides of the inside-out patches were washed with NaCl solutions, single-channel activity was generally not observed (Fig. 2a). When applied from the cytoplasmic side, cNMP reversibly suppressed (up to 70%) the integral current in a dose-dependent manner (4 of 26 patches). Micromolar concentrations of cGMP, cAMP, 8-Br-cGMP, 8-Br-cAMP, and millimolar concentrations of cCMP and 2', 3'-cGMP all decreased the patch currents (Fig. 2a, c).

FIG. 1 Whole-cell currents of TRC stimulated by bath application of tastants and patch-pipette-mediated introduction of peptides or cNMP. Recordings (a–d) began immediately after cell penetration. a, The patch pipette contained 1 mM ATP and 100  $\mu\text{M}$  peptide 1. b, The patch pipette was perfused with 20  $\mu\text{M}$  8-Br-cGMP in pseudo-intracellular (PI) solution. Downward arrow indicates the start of perfusion. c, TRC responses evoked by bath application of 5 mM saccharin (with or without IBMX) and 20  $\mu\text{M}$  amiloride. d, TRC responses evoked by bath application of 0.1 mM NC-01 (with or without IBMX) and 20  $\mu\text{M}$  amiloride. For both c and d, patch pipettes contained 1 mM ATP and 50  $\mu\text{M}$  GTP. e, TRC responses evoked by bath application of 0.1 mM NC-01 (NC-00274-01; ref. 10) in the absence (middle trace) and presence (bottom trace) of 0.4 mM IBMX. The upper trace is the time course of the stimulus (arbitrary units) (see Methods). The electrical artefacts in all three traces (subtracted in the other figures) were induced by injection of the solutions into the bath perfusion system and mark the start of perfusion. f, g, I–V curves from TRC that received peptide 1 (f) and 8-Br-cGMP (g) measured at moments 1–4 (of a, b respectively). In both cases, the I–V curves were nearly linear and crossed at about the same point, with an abscissa between –5 mV and 10 mV. h, I–V curves of the single taste-receptor cell (from e) that responded to both NC-01 and denatonium. Curves 1 and 2 for NC-01 (continuous lines) were measured at the points marked by the upward arrows in e; curves 1 and 2 for denatonium (circles) were measured at comparable points. All curves cross at ~9 mV. The I–V curves from another NC-01 responsive cell crossed at 11 mV (data not shown). Thus, both tastant- and cNMP-dependent currents are nearly voltage-independent and reverse at a potential close to zero. This suggests that both currents are mediated by the same ionic conductance(s); presumably non-selective cationic or anionic conductances (or both).

**METHODS.** Bullfrog (*Rana catesbeiana*) TRC were isolated as described<sup>23</sup>. TRC were identified by their typical bipolar shape<sup>23</sup> and patch-clamped (Axopatch 1D, Axon Instruments) on dendrite or soma. Data were acquired by Digi Data 1200 interface and analysed by the pClamp program (Axon Instruments). I–V curves were measured by

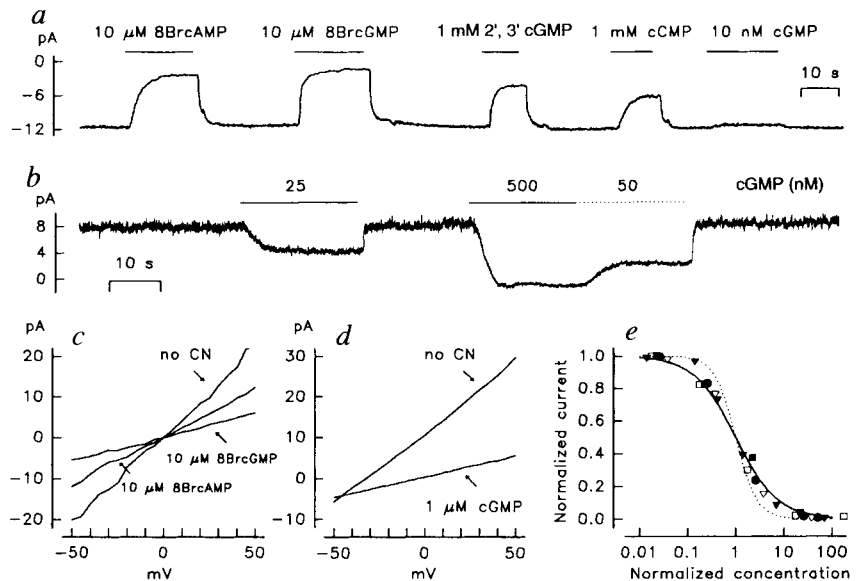


membrane polarization by both 200-ms voltage ramp and 50-ms voltage pulses of incrementally varying amplitudes. The recording chamber (50  $\mu\text{l}$ ) was perfused (8–40 bath volumes per min) by injection of solutions (0.2–1 ml) into the perfusion system. The solutions flowed by gravity and were sucked out of the chamber. TRC were stimulated by slow chamber perfusion. Patch pipettes were filled with PI solution (mM): KCl, 110;  $\text{MgCl}_2$ , 1; EGTA, 0.1; HEPES/KOH (pH 7.2), 10. Bath solution (mM): NaCl, 110; KCl, 1;  $\text{BaCl}_2$ , 10;  $\text{CaCl}_2$ , 1;  $\text{MgCl}_2$ , 1; HEPES/NaOH (pH 7.2), 10. 10 mM  $\text{BaCl}_2$  was added to suppress TRC potassium conductance<sup>11</sup> and identify presumptive effects of cNMP on other channels. Data were filtered at 1 kHz. The holding potential was –40 mV, except for the TRC in a, which was held at –30 mV; TRC WC recordings were most stable at this low holding potential. The time course of taste stimulus delivery was established by measuring the diffusion potential in the glass electrode tip evoked by saline perfusion. Two peptides (1 and 25) derived from rod  $\alpha$ -transducin's effector-interaction region and a control peptide (no. 66) from transducin's rhodopsin-interaction region were synthesized as described (refs 14, 15, and N. Spickofsky and R.F.M., unpublished). The dependence of frog rod PDE activity on peptides 1 and 25 gave  $\text{EC}_{50}$  values of 100 and 40  $\mu\text{M}$ , respectively (S. I. Zharikov, A. V. Kosolapov and S.S.K., unpublished). Patch pipettes were usually filled with PI solution containing 100  $\mu\text{M}$  of the particular peptide.

Inside-out patches were investigated with NaCl in the pipette and KCl in the bath: in 5 of 53 patches no prominent channel activity was observed, although integral outward current was suppressed by cNMP (Fig. 2*b*). The current inhibition was accompanied by a 30–70% decrease in patch conductance (Fig. 2*d*) and an evident fall in current noise (Fig. 2*b*), suggesting that cNMPs modulate the activity of some ion channels that are not resolved under our recording conditions. cNMP current

suppression (Fig. 2*a, b*) occurred in the absence of exogenous nucleotide triphosphates and in the presence of the protein-kinase inhibitor H8 (10  $\mu$ M), suggesting that suppression is independent of kinase-mediated phosphorylation. The inside-out patches were quite sensitive to cNMP: the half-maximal effective concentration ( $EC_{50}$ ) was 730 nM for 8-Br-cAMP (1 patch), 71 and 160 nM for cAMP (2 patches) and 16 and 36 nM for cGMP (2 patches) (Fig. 2*e*).  $K^+$  channels were observed in

FIG. 2 Cyclic nucleotides (cNMPs) suppress current in inside-out patches taken from the apical end of frog TRC. *a*, Effects of different cNMPs on the integral patch current at  $-20$  mV. Bath solution (mM): NaCl, 110;  $MgCl_2$ , 1; EGTA, 0.1; HEPES/NaOH (pH 7.2), 10. The pipette solution (mM): NaCl, 110; KCl, 1;  $CaCl_2$ , 1;  $MgCl_2$ , 1; HEPES/NaOH (pH 7.2), 10. Contaminant cGMP or cAMP may underlie the effects of 1 mM cCMP and 2', 3'-cGMP. Data were filtered at 500 Hz. *b*, Patch current variation at 0 mV induced by various cGMP concentrations. The pipette solution was as for *a*, the patch was perfused by PI solution (Fig. 1 legend). Data were filtered at 2 kHz. *c, d*, *I-V* curves of inside-out patches in the absence and presence of cNMP. Ion conditions are similar to those in *a* and *b*, respectively. *e*, Dose-response curves. The continuous and dotted lines correspond to the Hill equation with coefficients 1 and 2, respectively. The data indicate a Hill coefficient close to 1 ( $1.14 \pm 0.18$ ). The symbols correspond to: filled triangles, 8-Br-cAMP; filled squares and filled circles, cAMP; open squares and open triangles, cGMP. With a  $Na^+/K^+$  gradient across the patch membrane, cNMP suppressible currents reversed at about  $-50$  mV (see *d*), exhibiting predominantly a  $K^+$  selectivity, contrary to the observation that WC currents reverse at potentials close to zero when peptides or cNMP were introduced into the TRC (Fig. 1*f, g*). However, cNMP-suppressible  $Ca^{2+}$  influx (Fig. 3) may indirectly involve  $Ca^{2+}$ -dependent channels.  $Ca^{2+}$ -activated  $K^+$  and  $Cl^-$  channels have already been demonstrated in TRC<sup>11,25</sup>. Our preliminary observations indicate that a  $Ca^{2+}$ -activated  $Cl^-$  conductance may also operate in the frog TRC plasma membrane. These  $Ca^{2+}$ -activated channels may explain the discrepancy between



WC and inside-out patch recording.

**METHODS.** The dependence of integral current (at any voltage, because of the linear *I-V* curves) on the concentration (*C*) of cNMP in each experiment was fitted by the expression:  $I(C) = I_0 + I_1 / (1 + (C/K_{1/2})^n)$  to estimate the half-maximum concentration and Hill coefficient. The data are presented as the dependence of normalized current  $(I(C) - I_0) / I_1$  on normalized concentration  $(C/K_{1/2})$ .

FIG. 3 Indirect effects of cNMP on gating of  $Ca^{2+}$ -activated  $K^+$  (CAK) channels. *a*, Effects of cAMP and cGMP on inside-out patch current. *b, c*, Damped-out activity of three CAK channels evoked by the application of 100 nM cGMP (from *a*) on expanded timescales. *d*, The dependence of  $K^+$  channel activity on the concentration of free  $Ca^{2+}$  at 0 mV. The channel activity was referred to as  $I = iNP$ , where *I* is the mean  $K^+$  channel current, the number of channels and the open probability. The continuous line corresponds to the equation  $I/I_{max} = 1 / (1 + (K_{1/2}/[Ca])^n)$  with  $K_{1/2} = 340$  nM and  $n = 3$ . The concentration of free  $Ca^{2+}$  was adjusted by adding an appropriate amount of  $CaCl_2$  calculated according to standard equations<sup>26</sup>. Based on the  $Ca^{2+}$ -dependence and the single-channel *I-V* curve (data not shown) these  $K^+$  channels were identified as 74 pS  $Ca^{2+}$ -activated  $K^+$  (CAK) channels<sup>11</sup>. *e*, Effects of cGMP, EGTA and free  $Ca^{2+}$  on inside-out patch currents at 0 mV. Eight  $K^+$  channels operated in the patch.

**METHODS.** The patch-pipette filling solution (mM): NaCl, 110; KCl, 1;  $CaCl_2$ , 1;  $MgCl_2$ , 1; HEPES/NaOH (pH 7.2), 10. Bath solution for *a, b* and *c* (mM): KCl, 110;  $MgCl_2$ , 1; EGTA, 0.1; HEPES/KOH (pH 7.2), 10. For *d* and *e*, the bath solutions contained 1 mM EGTA. Data were filtered at 2 kHz. The activity of the CAK channel could be observed in the presence of 0.1 mM EGTA with no added  $Ca^{2+}$ . The total  $Ca^{2+}$  concentration in the patch volume should be about 40  $\mu$ M to maintain free  $Ca^{2+}$  at 100 nM, thereby evoking  $K^+$  channel activity (*d, e*). Our calculations indicate that  $Ca^{2+}$  diffusion by leakage (between the pipette wall and the membrane) into the volume of the patch is not capable of creating the required  $Ca^{2+}$  concentration near the patch membrane. For the  $K^+$  channels to compete with 0.1 mM EGTA, the  $Ca^{2+}$  ion must arise locally near  $K^+$  channels. Therefore,  $Ca^{2+}$  influx across the patch membrane is likely to be the source of the  $Ca^{2+}$  that activates the CAK channels.

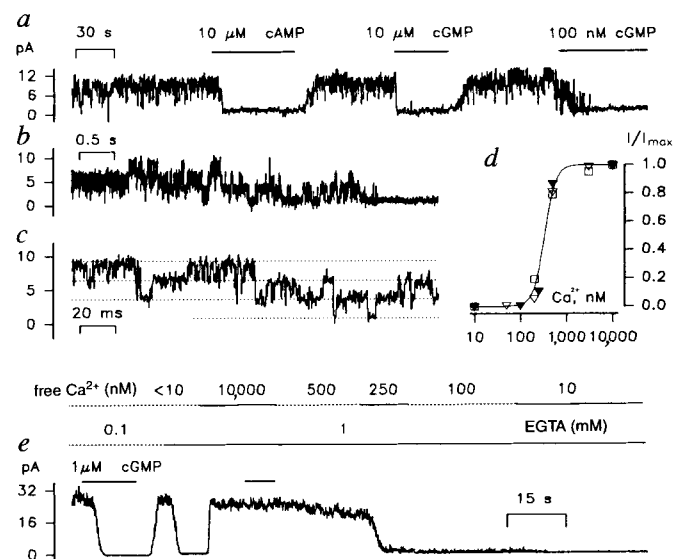
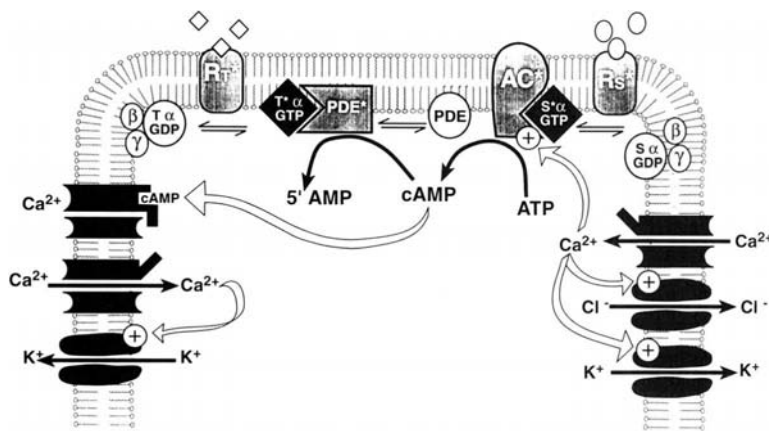


FIG. 4 Proposed taste transduction cascades.  $R_T^*$ , activated transducin-interacting receptor;  $T_\alpha$ -GDP, GDP-bound rod  $\alpha$ -transducin;  $T_\alpha$ -GTP, GTP-activated rod  $\alpha$ -transducin;  $R_S^*$ , activated  $G_S$ -interacting receptor;  $S_\alpha$ -GDP, GDP-bound  $G_S$   $\alpha$ -subunit;  $S_\alpha$ -GTP, GTP-activated  $G_S$   $\alpha$ -subunit; AC\*, activated adenylyl cyclase; PDE, cAMP phosphodiesterase; PDE\*, activated PDE. The following plasma membrane ion channels are shown:  $Ca^{2+}$ -activated  $K^+$  channel;  $Ca^{2+}$ -activated  $Cl^-$  channel; cNMP-suppressible cation channel. The  $R_S/G_S/AC$  pathway has been implicated in sweet transduction<sup>7-11</sup>. Activation of this pathway would elevate TRC cNMP levels, leading to phosphorylation and closure of basolateral  $K^+$  channels and TRC depolarization. The  $R_T/transducin/PDE$  pathway has been proposed for bitter transduction<sup>3,4,12</sup>. Activation of this pathway would decrease TRC cNMP levels, leading to activation of the cNMP-suppressible cation channel, TRC depolarization and  $Ca^{2+}$  influx.  $Ca^{2+}$  activation of  $K^+$  and  $Cl^-$  channels<sup>11,25</sup> as well as  $Ca^{2+}$  extrusion systems (i.e.  $Na^+/Ca^{2+}$  exchange) may participate in the TRC response. If  $Ca^{2+}$  activates frog TRC AC (as is the case with catfish taste tissue AC<sup>27</sup> and rat olfactory AC<sup>28</sup>) then AC in association with the cNMP-suppressible conductance may provide a negative feedback loop and underlie TRC adaptation. That both saccharin and NC-01 activate the  $R_T/trans-$



ducin/PDE pathway suggests either that frogs use this pathway for sweet or that these compounds are aversive (bitter) stimuli for the frog. Although both the  $R_T$  and  $R_S$  pathways are depicted they may be segregated into particular TRC subtypes.

the majority of inside-out patches with KCl in the bath: in 7 of 53 patches, cNMP (10  $\mu$ M) fully suppressed the activity of the known  $Ca^{2+}$ -activated 74 pS  $K^+$  (CAK) channels (Fig. 3a-d). The channels, initially active at 0.1 mM EGTA without added  $Ca^{2+}$ , were suppressed by cNMP (Fig. 3e); 1 mM EGTA also inhibited the activity of the CAK channels, whereas excess  $Ca^{2+}$  blocked the cNMP suppression of this  $K^+$  channel (Fig. 3e). These individual observations suggest that the effect of cNMP on gating of the CAK channel is indirect and results from the inhibition of the  $Ca^{2+}$ -permeable conductance of excised patches. This conductance is apparently the local  $Ca^{2+}$  source that activates CAK channels at 0.1 mM EGTA without added  $Ca^{2+}$ .  $Ca^{2+}$ -permeable cNMP-gated channels have been identified in several tissues<sup>22</sup>. Our data argue for the existence of a new type of cNMP-regulated  $Ca^{2+}$ -permeable channel: rather than cNMPs activating this channel, they suppress it.

Our preliminary data suggest that certain tastants (saccharin, for example) may induce  $Ca^{2+}$  elevation in frog TRC. Application of 10 mM saccharin evoked transient  $K^+$  channel activity in 3 of 33 cell-attached patches which persisted in all three inside-out patches; in 2 of 3 such patches, cNMP suppressed the activity of this CAK channel (data not shown). Voltage-gated  $Ca^{2+}$  channels have not been found in the frog TRC plasma membrane<sup>23</sup>; perhaps the cNMP-suppressible conductance is the major pathway of  $Ca^{2+}$  influx in frog TRC.

We propose the following model (Fig. 4): tastant-activated receptor activates transducin; activated transducin in turn activates cAMP-specific PDE. The ensuing drop in TRC cAMP concentration activates the cNMP-suppressible channel, leading to depolarization, increased  $Ca^{2+}$  influx and intracellular  $Ca^{2+}$ . The cascade may be turned off, as with the cGMP cascade in photoreceptors<sup>24</sup>, via receptor inactivation, hydrolysis of GTP by  $\alpha$ -transducin and  $Ca^{2+}$ -enhanced cNMP cyclase activity. Our model is supported by our observation that  $\alpha$ -transducin is present in mammalian TRC where it activates a PDE<sup>12</sup>. This model contrasts with proposals for sweet-taste transduction that implicate  $G_S$ , adenylyl cyclase, elevated cNMP and  $K^+$ -channel phosphorylation<sup>7-11</sup>. It may be that both of these pathways are used in different subsets of TRC. Alternatively, saccharin and NC-01 may stimulate bitter transduction in frog TRC. □

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## Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases

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CENTRAL to the role of p53 in cell regulation are its sequence-specific interactions with genes that control the cell cycle and apoptosis<sup>1,2</sup>. p53 response elements contain two or more copies of a somewhat promiscuous consensus sequence: 5'-XXXC(A,T)(T,A)GYYY-3' (where X is a purine and Y is a pyrimidine) (ref. 3). The sequence-specific DNA-binding region of p53 resides in its central conserved region<sup>4</sup>. Although this region itself is not known to be phosphorylated, the amino and carboxy termini of human p53 contain sites for phosphorylation by several protein kinases.

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We have examined the role of cyclin-dependent kinase (Cdk) shown previously to phosphorylate human p53 at serine 315 (ref. 5). We report here that p53 is efficiently and selectively phosphorylated by S and G2/M Cdk. Such phosphorylation markedly stimulates sequence-specific DNA binding by p53 and also causes a distinctive conformational change in p53 as revealed by partial protease analysis. Strikingly, Cdk phosphorylation also confers binding-site preference on p53. These data suggest a potential regulatory mechanism of p53 activity.

Immunopurified human p53 protein was phosphorylated *in vitro* by several immunopurified cyclin-dependent kinase complexes: cyclin D1/Cdk4, cyclin B/Cdc2, cyclin A/Cdk2 or cyclin E/Cdk2 (Fig. 1a). Purified Rb protein (Fig. 1a, bottom panel) was used to standardize the activity of the four cyclin-dependent kinases. When comparable amounts of these kinase complexes were used to phosphorylate p53 protein, marked differences were observed: cyclin B/Cdc2 and cyclin A/Cdk2 phosphorylated p53 efficiently. However, p53 was phosphorylated very poorly by cyclin E/Cdk2 and undetectably by cyclin D1/Cdk4. Importantly, two-dimensional tryptic phospho-peptide maps of  $^{32}$ P-labelled p53 phosphorylated *in vitro* by purified cyclin A/Cdk2 (Fig. 1c) or cyclin B/Cdc2 (results not shown) were virtually identical to those already published<sup>5</sup> and differed from those of p53 phosphorylated by casein kinase II at Ser 392 (ref. 6). This strongly suggests that the Cdk in the preparations used did not contain other contaminating kinases. Phosphorylation caused p53 to migrate through SDS gels more slowly than unphosphorylated p53 (Fig. 1b; compare lanes 2 and 3 or 6 and 7 with lane 1).

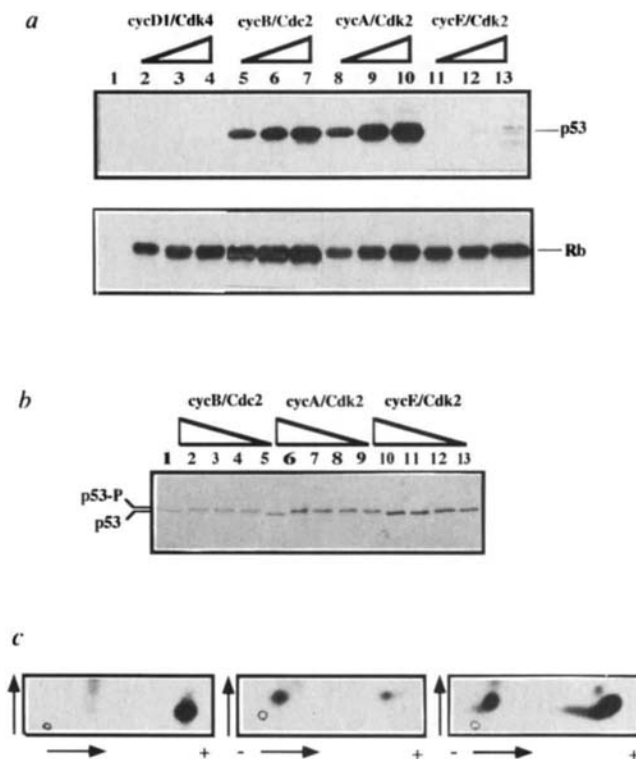
p53 binding to an oligonucleotide containing the GADD45 site, as measured by the electrophoretic mobility shift assay (EMSA), was examined after p53 was phosphorylated by each of the four cyclin-dependent kinase complexes *in vitro* (Fig. 2a). Phosphorylation of p53 by either cyclin B/Cdc2 or cyclin A/Cdk2 strongly stimulated binding to the GADD45 oligo-

nucleotide, whereas treatment with cyclin E/Cdk2 or cyclin D1/Cdk4 had little significant effect. The EMSA result therefore closely reflected the extent to which p53 was phosphorylated by cyclin-dependent kinases. Furthermore, the stimulation observed was caused by actual phosphorylation of the p53 protein because there was no increase seen when ATP was replaced by non-hydrolysable ATP (results not shown). The increased binding was sequence-specific and caused by p53 exclusively (Fig. 2b). Murine p53 DNA binding was also increased after Cdk phosphorylation, showing that this response is evolutionarily conserved (results not shown).

The enhancement of DNA binding by p53 after phosphorylation was also demonstrated in experiments in which cyclin-dependent kinases were immobilized on Sepharose beads (results not shown). This suggests that the p53 protein *per se* has been changed in some way by phosphorylation. To test the effects of Cdk kinase treatment on p53 structure, partial digestion of p53 by thermolysin after treatment with the cyclin-dependent kinases was performed in the absence and presence of ATP (Fig. 3). Phosphorylation by cyclin B/Cdc2 and cyclin A/Cdk2 caused significant differences in the partial protease digestion patterns in the presence of ATP compared with those seen in the absence of ATP. For example, there are several closely migrating cleavage products seen when ATP was absent (indicated by a bracket at the left in Fig. 3), but not when ATP was present. Moreover, two new cleaved p53 peptides are generated in the presence of ATP (indicated by two arrows at the right in Fig. 3) that are not detected in its absence. Both cyclin B/Cdc2 and cyclin A/Cdk2 caused the same alterations in thermolysin cleavage, whereas treatment of p53 by cyclin E/Cdk2 had only a subtle effect on the partial protease pattern. Cyclin D1/Cdk4 treatment did not cause any detectable change in the thermolysin products (results not shown). The altered protease sensitivity of phosphorylated p53 is strongly suggestive of a change in its

FIG. 1 Phosphorylation of p53 by cyclin A/Cdk2 and cyclin B/Cdc2 but not cyclin E/Cdk2 or cyclin D1/Cdk4 complexes. a, Human p53 protein (100 ng) or Rb protein (400 ng), as indicated at right, were incubated with increasing concentrations of cyclin D1/Cdk4 (lanes 2–4), cyclin B/Cdc2 (lanes 5–7), cyclin A/Cdk2 (lanes 8–10) or cyclin E/Cdk2 (lanes 11–13). Lane 1 contained a reaction mixture that lacked kinase. Mixtures were separated by 10% SDS–polyacrylamide gel and exposed to X-ray film. b, p53 protein (100 ng) was incubated with cyclin B/Cdc2 (lanes 2–5), cyclin A/Cdk2 (lanes 6–9) or cyclin E/Cdk2 (lanes 10–13), respectively. Lane 1 contained no kinase. Mixtures were separated on a 10% SDS–polyacrylamide gel and stained with silver. Positions of phosphorylated (p53-P) and unphosphorylated p53 (p53) are indicated at left. This mobility shift did not occur when ATP was replaced by non-hydrolysable ATP in the reaction mixtures (results not shown). c, Two-dimensional phospho-tryptic map of p53 phosphorylated by cyclin A/Cdk2 (left panel), casein kinase II (middle panel) or both cyclin A/Cdk2 and casein kinase II (right panel). Horizontal axis: electrophoretic separation; vertical axis: chromatographic separation.

METHODS. Human p53 was purified from baculovirus-infected SF9 cells by immunoaffinity procedures by using p53-specific PAb 421-Sepharose columns as previously described<sup>26</sup>. To isolate the different cyclin-dependent kinases, SF9 cells were co-infected with recombinant baculoviruses expressing either HA-tagged Cdk2 and cyclin A, HA-tagged Cdk2 and cyclin E, or HA-tagged Cdc2 and cyclin B. Cyclin kinase complexes were immunopurified from infected SF9 cell extracts by using the anti-HA antibody (12CA5) crosslinked to Sepharose by procedures essentially identical to those used for p53. Cyclin D1/Cdk4 was purified as described previously<sup>16</sup>. Rb protein was purified from extracts of SF9 cells that had been infected with a His-tagged Rb baculovirus by elution from Ni<sup>2+</sup>-NTA-Agarose (Qiagen) with 100 mM imidazole as described<sup>27</sup>. Phosphorylation reaction mixtures containing kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 100  $\mu$ M ATP) and 5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP were incubated for 30 min at 20 °C. For two-dimensional tryptic phosphopeptide mapping p53 was maximally phosphorylated by cyclin A/Cdk2 and/or casein kinase II, then subjected to SDS–PAGE and



transfer to a nitrocellulose filter. The phosphorylated p53 protein on the filter was then digested with trypsin, further processed and subjected to electrophoresis in the first dimension and chromatography in the second dimension as described<sup>28</sup>.



conformation, which may be responsible for the enhanced sequence-specific DNA-binding ability of p53 by Cdk.

Although most of the known p53 sites fit the rather loose p53 consensus binding sequence, they vary considerably. The flexibility of the different p53 binding sites suggests an additional level of regulation of p53 that may be related to the Cdk-induced altered conformation of p53. Thus, it is possible that phosphorylation of p53 might alter its relative ability to bind to different sites. To test this, p53 that was or was not phosphorylated *in vitro* by cyclin kinase complexes was tested for binding to different oligonucleotides, each containing different p53-binding sites (Fig. 4). Immunopurified p53 proteins were first phosphorylated by the cyclin-dependent kinases, again standardized by their ability to phosphorylate Rb protein. EMSA reaction mixtures containing equal amounts of each of the labelled oligonucleotides were then added to the kinase mixtures (Fig. 4a). Phosphorylation by cyclin B/Cdc2 and cyclin A/Cdk2 stimulated the binding of p53 to the WAF1/p21/Cip1 binding site to an even greater extent (15–30-fold) than to the GADD45 site (10–15-fold). Remarkably, however, the binding to the RGC

site was stimulated only by about threefold. Moreover, p53 binding to oligonucleotides containing two other sites, MCK and SV40, was virtually unaffected by phosphorylation by the cyclin kinases (summarized graphically in Fig. 4b). There are no obvious residues that distinguish the 'good' from the 'bad' sites. However, a common feature of the GADD45 and WAF1/p21/Cip1 sites (to which binding by p53 was preferentially stimulated) that is not shared by the other sites is their strongly palindromic nature. Whether that is the reason for their being differentially bound by phosphorylated p53 is now under investigation. It should be noted that phosphorylation of p53 by another purified protein kinase, casein kinase II, also activated DNA binding as previously reported<sup>7</sup>, and also preferentially stimulated binding to the WAF1/p21/Cip1 and GADD45 oligonucleotides (results not shown). However, casein kinase II caused neither the electrophoretic mobility shift nor alterations in thermolysin cleavage of p53 that resulted from its phosphorylation by Cdk (results not shown). Although this indicates that the conformational change induced by Cdk is not necessary for altering its DNA-binding properties, it suggests that the two

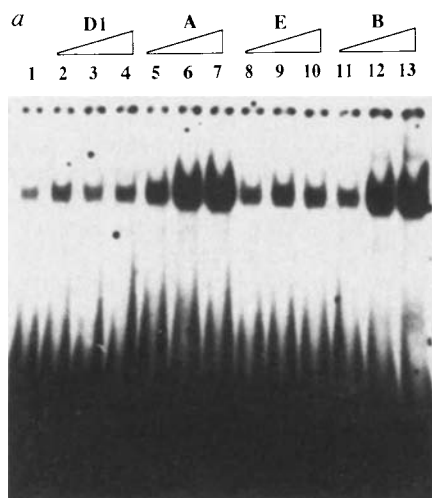
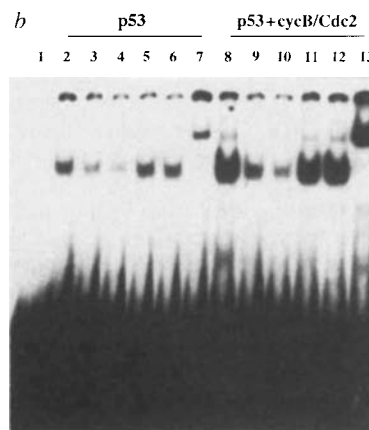


FIG. 2 Stimulation of sequence-specific binding of p53 to DNA by phosphorylation of p53 by cyclin B/Cdc2 and cyclin A/Cdk2. **a**, Human p53 (100 ng) was phosphorylated by the same range of quantities of cyclin kinase complexes (D1, cyclin D1/Cdk4; A, cyclin A/Cdk2; E, cyclin E/Cdk2; B, cyclin B/Cdc2) as in Fig. 1 and then tested for binding to a <sup>32</sup>P-labelled oligonucleotide containing the GADD45 site by EMSA. **b**, Human p53 (100 ng, lanes 2–13) was incubated in kinase buffer without (lanes 2–7) or with (lanes 8–13) cyclin B/Cdc2 kinase complex followed by incubation in DNA-binding buffer containing the <sup>32</sup>P-labelled GADD45 oligonucleotide in the presence of 20-fold (lanes 3 and 9) or 40-fold (lanes 4 and 10) molar excess of unlabelled wild-type GADD45 oligonucleotides, or 20-fold (lanes 5 and 11) or 40-fold (lanes 6 and



12) excess of unlabelled mutant GADD45 oligonucleotides. The reaction mixtures in lanes 7 and 13 also contained p53 monoclonal antibody, PAb 1801; there were no proteins in lane 1.

**METHODS.** p53 was incubated in buffer containing 50 mM HEPES, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT and 100 μM ATP for 30 min at 20 °C with or without cyclin B/Cdc2. DNA-binding buffer (25 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM spermidine, 0.25 mM DTT, 0.025% NP40, 4 μg BSA, 100 ng poly(dI:dC) containing 1 ng of <sup>32</sup>P-end-labelled GADD45 (ref. 18) oligonucleotide (5'-AATTCTCGAGCAGAACATGTCT-AAGCATGCTGGGCTCGAG-3') and either unlabelled wild-type GADD45 or mutant GADD45 (5'-AATTCTCGAGCAGAAAATTCTAAGAATTCTGGGCTCGAG-3') oligonucleotides or 1 μg PAb 1801 monoclonal antibody, as indicated, were then added to each tube and mixtures incubated for a further 30 min on ice. Reaction mixtures were then separated on a 4% native polyacrylamide gel and exposed to X-ray film.

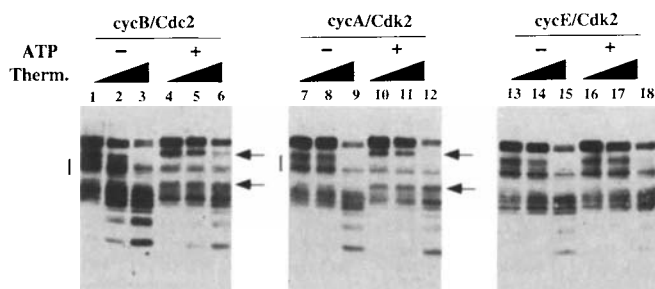


FIG. 3 Alteration of the protease sensitivity of p53 after its phosphorylation by cyclin B/Cdc2 and cyclin A/Cdk2. p53 was incubated in kinase buffer with cyclin B/Cdc2 (lanes 1–6), cyclin A/Cdk2 (lanes 7–12) or cyclin E/Cdk2 (lanes 13–18) in the absence (lanes 1–3, 7–9 and 13–15) or presence (lanes 4–6, 10–12 and 16–18) of ATP. Proteins were then treated with thermolysin in quantities of 100 ng (lanes 1, 4, 7, 10, 13 and 16), 200 ng (lanes 2, 5, 8, 11, 14 and 17) or 400 ng (lanes 3, 6, 9, 12, 15 and 18).

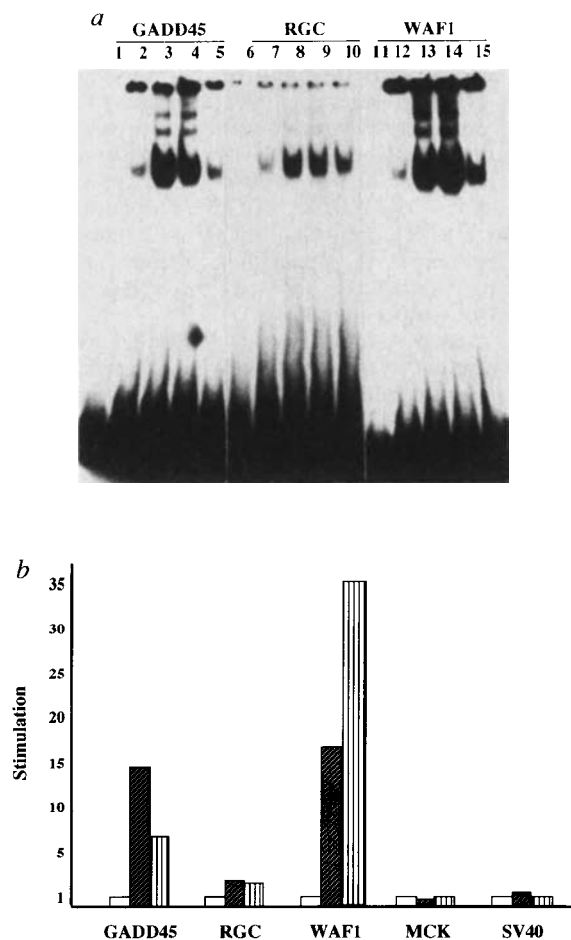
**METHODS.** p53 (100 ng) was incubated in kinase buffer as in Fig. 1 in the absence or presence of 100 μM ATP for 40 min on ice. Thermolysin (Therm.) (Boehringer Mannheim) was added in the indicated quantities and incubation continued for another 30 min at 20 °C. Reaction mixtures were then separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. p53 peptides were revealed by probing with a mixture of p53 antibodies, PAb 1801, 240 and 421, and subsequent developing with ECL solutions (Amersham).

kinases exert their stimulatory effect on p53 by different modes.

Little is known about the function of the p53 Cdk site in cells. It was reported that p53 mutated at this site is capable of inducing growth arrest with the same efficiency as wild-type p53 (refs 8–11). By contrast, mutation of this site caused increased stability of the p53 protein<sup>12</sup>. However, experiments testing p53

transfected into cells usually involve constructs in which p53 is expressed at significantly higher levels than normally found in cells. Cdk phosphorylation may be crucial only when p53 quantities are limiting. The various cyclin complexes we have examined are each prevalent at a different stage of the cell cycle<sup>13–15</sup>. Our data showing that cyclin A/Cdk2 and cyclin B/Cdc2 but not cyclin E/Cdk4 and cyclin D1/Cdk4 kinase complexes phosphorylate p53 provide further evidence for significant differences in substrate specificity of Cdk kinase imparted by different cyclins<sup>16</sup>. Because cyclin D1 and cyclin E are most abundant in early and late G1 respectively, they also correlate nicely with previous observations that p53 is underphosphorylated at the Cdk site in G1 (refs 5, 17). One must, however, align these observations with the fact that p53 functions as a DNA-damage-sensitive checkpoint factor that arrests cells primarily in G1 (refs 18, 19). Clearly, the potency of this pathway, and in some cases (as when apoptosis is induced) its irreversibility, require tight restrictions on p53 activity. Thus, it might be disadvantageous to have a hyperactive p53 in normal cycling cells in G1. It is therefore intriguing that a potential new target of p53, cyclin G, that is induced upon DNA damage, has been identified<sup>20</sup>. Whether complexes containing cyclin G are capable of phosphorylating and stimulating the binding of p53 to DNA will therefore clearly be of great interest. The fact that there is good evidence that p53 is involved in blocking cells at G2/M (refs 21–23), as well as in G1, is consistent with our results. Indeed, the WAF1/p21/Cip1 cyclin kinase inhibitor that is induced by p53 is found in several cyclin kinase complexes including those prevalent in the S and G2 phases<sup>24</sup>. A regulatory loop may be operating as follows: cells overexpressing Cdks would generate an activated p53 that would induce p21, which in turn would downregulate both the Cdk complex and p53.

Finally, although there are multiple examples documenting phosphorylation-induced stimulation of DNA-binding proteins<sup>25</sup>, the results presented here represent the first example of a eukaryotic protein whose ability to bind to various sites is differentially affected by phosphorylation. Our results therefore suggest that the loose p53 consensus sequence may provide an additional level of regulation for p53 activity. □



**FIG. 4** Alteration of the DNA-binding specificity of p53 after phosphorylation by cyclin B/Cdc2 and cyclin A/Cdk2. **a**, p53 protein (100 ng, lanes 2–5, 7–10 and 12–15) was first phosphorylated by cyclin B/Cdc2 (lanes 3, 8 and 13), cyclin A/Cdk2 (lanes 4, 9 and 14) or cyclin E/Cdk2 (lanes 5, 10 and 15) and then bound to DNA as in Fig. 2. Reaction mixtures included <sup>32</sup>P-labelled oligonucleotides containing the GADD45 site (lanes 1–5), the RGC site (lanes 6–10) or the WAF1/p21/Cip1 site (lanes 11–15). There were no proteins in lanes 1, 6 and 11. **b**, Graphic representation of the data shown in **a** (stimulation shown as a multiple of unphosphorylated p53) with additional results in which oligonucleotides containing the MCK and SV40 sites were tested in the same way. Open bars represent unphosphorylated p53. Diagonally hatched bars represent cyclin B/Cdc2 phosphorylated p53 and vertically hatched bars represent cyclin A/Cdk2 phosphorylated p53. **METHODS.** p53 protein (100 ng) was first phosphorylated by cyclin-dependent kinase complexes as indicated and then bound to DNA as in Fig. 2. Reaction mixtures contained <sup>32</sup>P-labelled oligonucleotides (1 ng) with the GADD45, RGC (ref. 29) (5'-TCGAGTTGCCTGGACTTGGC-TGGCCTTGCCTTC-3'), WAF1/p21/Cip1 (ref. 30) (5'-AATTCTCGAGGAAC-ATGTCCCAACATGTTGCTCGAG-3'), MCK (ref. 29) (5'-TCGAGTGGCAAG-CCTATGACATGGCCGGGCGCTGCTCTCTGCTCTGACCT-3') and SV40 (ref. 29) (5'-TCGAGCCATGGGCGGAGAATGGGCGGAAGTGGG-CGGAGTTAGC-3') sites as indicated. Reaction mixtures were then separated on a 4% native polyacrylamide gel and exposed to X-ray film. Results in **b** were measured with a Phosphorimager using Image-Quant software.

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# Structure of influenza virus haemagglutinin complexed with a neutralizing antibody

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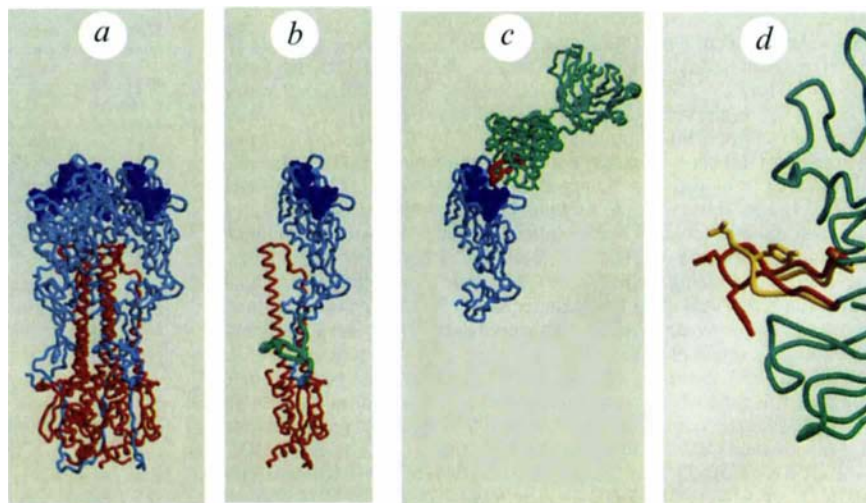
**HAEMAGGLUTININ (HA) is the influenza surface glycoprotein that interacts with infectivity-neutralizing antibodies. As a consequence of this immune pressure, it is the variable virus component, which is important in antigenic drift, that results in recurrent epidemics of influenza. We have determined the crystallographic structure of a complex formed between the antigen-binding fragment (Fab) of a neutralizing antibody and the membrane-distal domain ('HA top') of a HA subunit prepared from HA in its membrane-fusion-active conformation. A dramatic change is seen in the structure of the Fab-combining site on complex formation. Our results indicate that neutralization of infectivity by this antibody involves the inhibition of receptor binding, and demonstrate how influenza virus can maintain its conserved receptor-binding site despite the immune selective pressure for change in this region of the molecule; they also contribute to a complete description of the endosomal pH-induced fusion-active HA structure.**

HA is a trimer of identical subunits, each of which contains two polypeptides, HA<sub>1</sub> and HA<sub>2</sub>. HA<sub>1</sub> primarily forms a membrane-distal globular domain, HA<sub>2</sub> a central helix-rich stem structure<sup>1</sup>. HA has two functions in virus replication; it binds

viruses to cell-surface glycoconjugates by recognizing terminal sialic acid residues of carbohydrate side chains, and, following endocytosis of bound virus, it mediates the fusion of virus and endosomal membranes required for transfer of the virus genome-transcriptase complex into the cell<sup>2</sup>. Binding to sialic acid receptor involves a shallow pocket of conserved amino acid residues near the membrane-distal tip of each subunit and has been defined by crystallographic studies of receptor analogue-HA complexes<sup>3</sup>. Membrane fusion by HA is activated at endosomal pH, between pH 5.0 and 6.0, in a process that involves extensive refolding and rearrangement of the molecule. The fusion pH structure of HA has been studied crystallographically<sup>4</sup>, using soluble fragments (TBHA<sub>2</sub>) prepared by proteolysis of fusion pH-induced HA aggregates, first to release the globular membrane-distal domains ('HA tops') and then to render the aggregated trimeric coiled-coils soluble by removing their amino-terminal hydrophobic 'fusion peptides'. 'HA top' prepared in this way contains HA<sub>1</sub> residues 28–328 and is monomeric<sup>5</sup>. Its secondary structure is indistinguishable by circular dichroism from that deduced in the intact molecule, and it binds anti-HA monoclonal antibodies other than those that are known to interact near the trimer interface<sup>6,7</sup>.

We have used 'HA top' released by *LysC* digestion of the fusion-pH form of X-31 HA (H<sub>3</sub> subtype) to prepare the complex analysed in this study. The structure determination at 3.3 Å resolution is described in Table 1. 'HA top' is monomeric in the crystal as observed in solution, and substantially retains the structure it adopts in native HA (Fig. 1). Two lines of evidence indicate that rearrangement of the HA membrane-distal domain is required for fusion. First, mutations in the membrane-distal trimer interface alter the pH of fusion<sup>8</sup>. Second, introduction by site-specific mutation of disulphide bonds that crosslink the membrane-distal domain prevents the low-pH-induced changes in HA structure and prevents fusion<sup>9</sup>. The conservation of the structure of 'HA top' that we observe indicates that the rearrangements in the membrane-distal region of HA that are required for membrane fusion activity primarily involve dissociation of the trimeric membrane-distal globular domain. 'HA tops' are tethered to the rest of the molecule in the fusion pH conformation by a disulphide bond near the N terminus of HA<sub>1</sub> (HA<sub>1</sub> 14 to HA<sub>2</sub> 137). Two fragments of HA<sub>1</sub>, residues 1–27 in the TBHA<sub>2</sub> structure<sup>4</sup> and residues 28–43 analysed here, are

FIG. 1. Ribbon diagrams of X31 BHA and of the X31 'HA top'—HC19 Fab complex. *a*, X31 BHA trimer. The HA<sub>1</sub> chains are coloured blue and the HA<sub>2</sub> chains are red. The receptor-binding sites are shown in dark blue CPK representation. *b*, X31 BHA monomer. HA<sub>1</sub> residues 17–43 that link the two fragments used to analyse the structure of the HA molecule in its fusion-pH conformation, 'HA top' and TBHA<sub>2</sub>, are shown in green. *c*, X31 'HA top'—HC19 Fab complex. The whole Fab (in green) is in readily interpretable electron density in the final map, except one loop remote from the combining site. In the 'HA top' part of the complex, only residues 43–309 are well defined and shown here (in blue). This 'HA top' part of the complex retains the structure it adopts in BHA: the root mean square deviation of *C<sub>α</sub>* between this structure of 'HA top' and the corresponding part of HA<sub>1</sub> in BHA is 0.69 Å. The Fab H3 CDR loop (in red) is seen protruding from the Fab into the HA receptor-binding site (dark blue CPK) and blocking its access. *d*, Close-up of the structure of the Fab-combining site, showing the change in H3 CDR loop conformation on Fab—HA complex formation. The H3 loop in the Fab uncomplexed structure (yellow) has been superimposed on the corresponding loop in the complex (red). The side chains of Tyr H102 and Asp H103, which differ



most in the two structures, are also displayed. Residues Tyr H102 and Asp H103 interact with X31 HA in the complex, which would not have been possible in the H3 conformation of uncomplexed HC19. All figures were generated with Molscript<sup>30</sup>.



TABLE 1 Crystallographic data and refinement statistics

|                                               | HA VA19         | HA X31 Top<br>Fab HC19 | HA X31 Top<br>Fab HC19 |
|-----------------------------------------------|-----------------|------------------------|------------------------|
| Data collection statistics                    |                 |                        |                        |
| Number of crystals                            | 6               | 1                      | 3                      |
| Maximum resolution (Å)                        | 3.4             | 4.0                    | 3.3                    |
| Observations                                  | 131,577         | 31,960                 | 106,437                |
| Unique reflections                            | 54,991          | 9,712                  | 17,678                 |
| Completeness (%)                              | 85              | 93.5                   | 98.0                   |
| $R_{\text{sym}}^*$                            | 0.078           | 0.084                  | 0.093                  |
| Refinement statistics                         |                 |                        |                        |
| $R_{\text{cryst}}^\dagger$ (resolution range) | 0.208 (7–3.4 Å) | †                      | 0.169 (7–3.3 Å)        |
| Deviations from ideal geometry:               |                 |                        |                        |
| Bond lengths (Å)                              | 0.016           | †                      | 0.012                  |
| Bond angles (degrees)                         | 3.0             | †                      | 1.8                    |

X31 virus (H3N2) was grown in embryonated eggs and purified as described previously<sup>19</sup>. Virus was incubated at pH 5.05 (37 °C, 10 min), then treated at pH 7.2 (20 °C, 2 h) with 0.15 µg of endoproteinase Lys C (Boehringer) per 1 mg of virus. The reaction was stopped by the addition of soybean trypsin inhibitor in excess. 'HA top' was separated from the virus by centrifugation, its concentration adjusted to 0.5 mg ml<sup>-1</sup> in 100 mM phosphate buffer, pH 6, 30 mM EDTA, and it was deglycosylated for 36 h at 37 °C with a mixture of N-glycosidase F (Boehringer), using 40 U per mg of 'HA top', and endoglycosidase H (Boehringer), using 50 mU per mg of 'HA top'. Fab fragment HC19 purified as described<sup>20</sup> was then added in excess and the 'HA top'-Fab complex was purified by size-exclusion chromatography. Crystals of the complex were obtained by the hanging-drop procedure using 1-ml wells containing 150 mM NaCl, 1 mM ZnCl<sub>2</sub>, 50 mM MES, pH 6, and 4 µl drops containing a protein solution (5–10 mg ml<sup>-1</sup>) in 75 mM NaCl, 0.5 mM ZnCl<sub>2</sub>, 25 mM MES, pH 6. Crystals belong to space group P6<sub>3</sub>22 with cell dimensions  $a = b = 85.5$  Å,  $c = 515$  Å and diffract at least to 2.5 Å resolution; nevertheless, the occurrence of a very long axis and the need to achieve spot separation have so far precluded data collection to a resolution below 3.3 Å. A data set to 4 Å resolution was collected at the LURE synchrotron (Orsay, France) and used for molecular replacement. The reflections were indexed with DENZO (Z. Otwinowski, personal communication), integrated with MOSFLM<sup>21</sup> and scaled with programs of the CCP4 suite<sup>22</sup>. Molecular replacement calculations were performed with package AMORE<sup>23</sup>, using coordinates of X31 BHA (HA solubilized from virus by bromelain digestion<sup>1</sup>) and of HC19 Fab<sup>14</sup> as search objects. Data collection to 3.3 Å resolution was performed at the ESRF (Grenoble, France) and DESY (Hamburg, Germany) synchrotron sources. Data processing was performed as above and reflections in the 7–3.3 Å resolution shell (with  $F_{\text{obs}} > 2 \sigma(F_{\text{obs}})$ ) were used for alternate rounds of model building with FRODO<sup>24</sup> and refinement of atomic coordinates (using XPLOR<sup>25</sup>). The stereochemistry of the current model was verified with PROCHECK<sup>26</sup>. Data collection for HA VA 19 was performed at the DESY synchrotron and data were processed with MOSFLM<sup>21</sup>. The structure was solved as in ref. 16.

Coordinates of HA VA19 and C $\alpha$  coordinates of the current model for the complex 'HA X31 top'-Fab HC19 will be deposited at the Protein Data Bank.

\*  $R_{\text{sym}} = \sum_i |I_i - \langle I \rangle| / \sum_i \langle I \rangle$ , where  $I_i$  is the intensity measurement for reflection  $j$  and  $\langle I \rangle$  is the mean intensity for multiply recorded reflections.

†  $R_{\text{cryst}} = \sum_i |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_i |F_{\text{obs}}|$ .

‡ Data set used for molecular replacement only.

mainly disordered, suggesting that the polypeptide tether that links 'HA top' to the rest of the molecule is flexible at fusion pH. This is consistent with electron microscopy of HA in the fusion-pH conformation<sup>10</sup>. The fusion-pH HA structure therefore appears to result from a dramatic structural rearrangement of HA<sub>2</sub> together with the unfolding and membrane-distal extension of the N-terminal strand of HA<sub>1</sub> that tethers dissociated but otherwise structurally unchanged 'HA tops' to the rest of the molecule.

The structure of the Fab fragment prepared from antibody HC19 and the HA-binding specificity of the antibody have been reported elsewhere<sup>11,12</sup>. The structure of the HC19-combining site changes significantly on complex formation, as in other antigen-antibody or hapten-antibody complexes<sup>13</sup>. The change in the distal tip of the H3 complementarity-determining region (CDR) is particularly dramatic, with some atoms differing in location by more than 10 Å between the complexed and uncomplexed structures (Fig. 1d). These changes allow 4 of the 10 hydrogen bonds in the interface to be formed; they have precluded correct prediction of the HA-Fab complex structure by a docking algorithm using the known structures of uncomplexed X-31 HA and HC19 Fab (ref. 14).

The general features of the interface in the HA-Fab complex are within the ranges found for other protein-protein complexes<sup>15</sup>; the buried surface area is 1,250 Å<sup>2</sup> compared with a reported range of 1,600 ± 350 Å<sup>2</sup>; the number of intermolecular hydrogen bonds identified is 10 compared with the reported average of 10. There is a cavity in the interface which has an opening to bulk solvent and is presumably filled by disordered water molecules. On the HA side of the interface, residues largely buried upon complex formation include amino acids at positions 134, 136, 153, 155 and 194 of HA<sub>1</sub>, which contribute main-chain

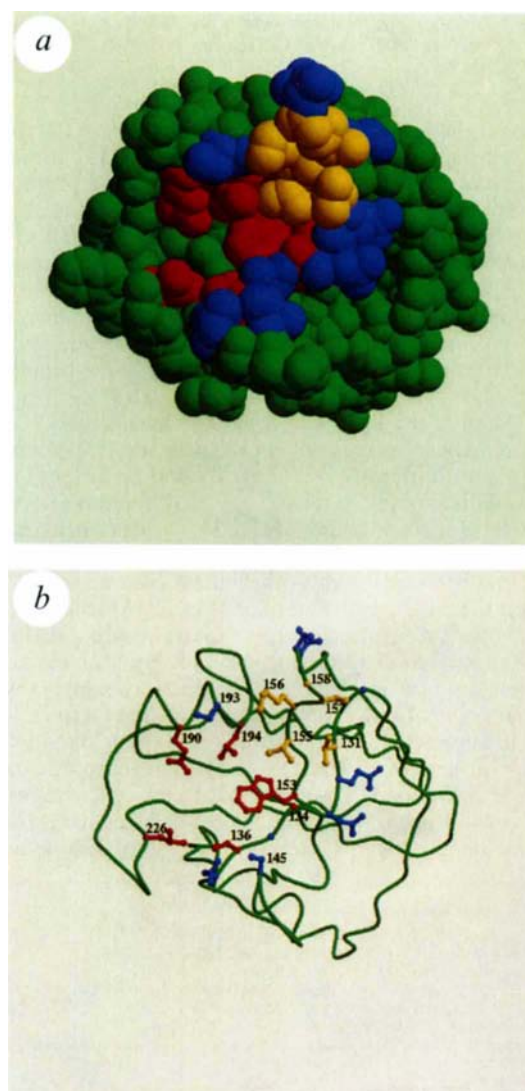


FIG. 2 Residues of X31 HA in the interface of the HC19-Fab-X31-HA complex. The two views are seen in the same orientation, perpendicular to an average plane of the interface. Residues of HA that bury some of their surface in the complex have been coloured according to their classification in Table 2. Amino acids at positions where mutations have been identified that allow escape from neutralization by HC19 are in yellow; all except Thr 155 are on an edge of the interface. The centre of the interface consists mostly of residues that belong to the receptor binding site; they are in orange. Other residues in the interface are in blue; mutations of these residues do not affect HC19 binding to X31 HA in single-amino-acid mutants that have been isolated. a, CPK model of the interface embedded in a CPK representation of 'HA top' (green). b, 'HA top' is modelled as a green ribbon on which the side chains of residues in the HA-Fab interface are grafted.



TABLE 2 Correlation of mutant properties with accessible surface area of residues in the HC19-HA interface

| HA*<br>residue                           | 131<br>Thr→Ile | 134<br>Gly | 136<br>Ser | 145<br>Ser→Asn | 153<br>Trp | 155§<br>Thr→Tyr | 156  <br>Lys→Glu | 157<br>Ser→Leu | 158<br>Gly→Glu | 190<br>Glu | 193<br>Ser→Arg | 194<br>Leu | 226<br>Leu→Gln |
|------------------------------------------|----------------|------------|------------|----------------|------------|-----------------|------------------|----------------|----------------|------------|----------------|------------|----------------|
| Mutant neutralized by HC19†              | —              | NA¶        | NA         | +              | NA         | —               | ±☆               | —              | —              | NA         | +              | NA         | +              |
| A.s.a.‡ free structure (Å <sup>2</sup> ) | 69             | 17         | 18         | 40             | 20         | 20              | 62               | 42             | 38             | 45         | 57             | 43         | 43             |
| A.s.a. in complex (Å <sup>2</sup> )      | 16             | 0          | 0          | 34             | 5          | 4               | 25               | 1              | 0              | 22         | 28             | 1          | 28             |

\* The position in HA<sub>1</sub> is given. When two residues are given, a mutant with the corresponding mutation has been identified at this position. All mutations are in a single amino-acid variant except where indicated.

† +, Mutant bound by HC19; —, mutant escaping recognition by HC19 (measured by ELISA and inhibition of haemagglutination). Mutants at positions 131, 157 and 158 were selected by growing X31 virus in the presence of HC19 antibody and escape neutralization by HC19.

‡ A.s.a., accessible surface area, calculated according to the algorithm of Shrake and Rupley<sup>27</sup>, using a 1.4-Å-radius probe. Only side-chain contributions are considered (including Cys) and all HA residues are listed where this contribution is different in the free HA and in the complex structure and where mutant data are available.

§ This mutation is one of the ten amino-acid changes in strain A/Eng/42/72 HA (ref. 28) compared with X31 HA, but is the only one of a residue located in the X31-HA-HC19 interface. HC19 does not inhibit haemagglutination by A/Eng/42/72 virus<sup>29</sup>.

|| The amino acid at 156 does not change to a bulkier one and is part of a double mutant. The other change (193 Ser→Arg) may contribute to the lowering of HC19 affinity, because the two residues, whose C $\alpha$ s are distant by 8.1 Å, may reorient to allow their side chains to form a salt bridge.

¶ NA, not applicable; the residue is invariant (see text).

☆ The infectivity in M.D.C.K. cells of this double mutant is neutralized only at HC19 concentrations 100 times greater than those required to neutralize X31 virus.

atoms or conserved side chains to the formation of the right side (134, 136), the rear (155, 194) and the bottom (153) of the sialic acid receptor-binding site<sup>3</sup>. Despite the concave shape of this part of HA<sup>1</sup>, extensive contacts to the right half of the receptor-binding site are made by the Fab; 167 Å<sup>2</sup> of 315 Å<sup>2</sup> solvent-accessible surface in the receptor-binding site is buried on HA-HC19 complex formation. These contacts involve the Fab H3 CDR loop and would prevent attachment of HA to its sialylated receptor, as observed in the inhibition by HC19 and HC19 Fab of X-31-virus-mediated haemagglutination (unpublished results).

The region of the X-31 HA recognized by antibody HC19 has also been mapped by selecting and sequencing HA mutants that escape neutralization by the antibody and by determining the relative affinities of HC19 for various X-31 mutants selected with other monoclonal antibodies (Table 2). The conserved sialic acid receptor-binding site fills the centre of the HA-Fab interface and all the mutated residues that affect binding of HC19 are found on the edge of this interface (Fig. 2). Most of the mutations involve substitution with a bulkier residue; all of them are on the surface of HA, as is generally true for antigenically significant residues in HA<sup>2,16</sup>. They most probably cause only local changes in HA structure and this was shown to be the case for the amino-acid substitution 157 Ser→Leu, by solving the structure of the mutant HA (Table 1); changes in this structure relative to wild-type HA were limited to the replacement of the serine by the leucine side chain. Local changes have also been shown to be all that is involved in a mutant HA selected by

another neutralizing antibody<sup>17</sup>, although other possibilities exist<sup>18</sup>.

A comparison of the molecular locations of variable amino-acid residues in HA and accessible surface area in the HA-Fab complex shows that at all variable positions in the interface where amino-acid side chains have a very small solvent-accessible surface area (less than 5 Å<sup>2</sup>), a mutation that abrogates neutralization by HC19 has been identified (Table 2). Reciprocally, at positions where side chains maintain a large solvent-accessible surface in the interface (more than 20 Å<sup>2</sup>) and where substitutions bulkier than the wild-type residue have been identified, the mutants are bound by HC19 and do not escape neutralization. These results suggest that steric hindrance in the complex with a neutralizing antibody is the major molecular basis for selection of HA mutants that escape neutralization. The only apparent constraint under which this mechanism operates is that of conserving residues required for virus viability; in the HC19:HA interface, unchanged amino-acid residues form the sialic acid receptor-binding site. The conservation of the receptor-binding site residues is made possible even under the selective pressure of numerous antibodies that recognize this region of HA because the greater surface area of an antibody footprint, by comparison with the surface area of the receptor-binding site (600–1,000 Å<sup>2</sup> versus 315 Å<sup>2</sup>) ensures that all HA-antibody interfaces contain HA residues not required for the receptor-binding function. Mutations at these positions prevent antibody binding and yield mutant viruses that escape neutralization of infectivity. □

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# Nature's sister journals in review

Articles flagged here look into a possible way to control proliferation of cancer cells, the origins of some common birth defects, and mechanisms of catalysis of a tRNA synthetase and two aminotransferases.

## Enzymes as adaptors

ANY information store is useless unless it can be decoded. In the case of the genome of living systems, transfer RNA is the adaptor molecule that interprets the triplet code of the messenger RNA and converts it into the strings of amino acids that form proteins. This adaptor function is achieved by dual

recognition of a single triplet codon in mRNA and of a particular amino acid that is attached to the other end of the molecule. Because codon-anticodon pairing is independent of the amino acid attached to the tRNA, the enzymes that charge the tRNAs with activated amino acids — the tRNA synthetases — play a critical role in determining the fidelity of the decoding process.

The structure of the most complex of the tRNA synthetases, phenylalanyl-tRNA synthetase (PheRS), a class II synthetase, is presented by Mark Safo and colleagues in this month's issue of *Nature Structural Biology* (page 537). The overall shape of the tetrameric enzyme,  $(\alpha\beta)_2$ , is reminiscent of a leatherback turtle (see figure), with the two large flippers being formed by N-terminal portions of the two  $\beta$ -subunits. As suspected, the  $\alpha$ -subunit contains the characteristic class II catalytic domain. The  $\beta$ -subunit possesses an intriguing ensemble of folds. A non-functional duplication of the catalytic domain seems to help maintain the  $\alpha$ -subunit in an appropriate conformation for catalysis. There are also the equivalents of an anticodon-loop-recognition domain, 'rimming loops', which mediate interaction with tRNAs, and an RNA-binding domain (similar to that of the U1A spliceosomal protein) which may be involved in binding to the tRNA acceptor stem. An SH3-like domain and a helix-turn-helix domain similar to that in the

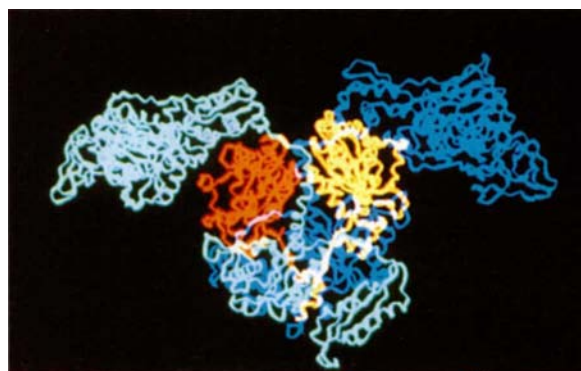
catabolite gene activator protein are also present, but their roles are as yet unclear.

Understanding the workings of enzymes such as PheRS will allow scientists to manipulate their catalytic activity. As part of this continuing effort, Johan Jansson and colleagues are probing the molecular basis of enzyme-substrate specificity in aspartate

aminotransferase (AspAT) and tyrosine aminotransferase (TyrAT) (page 548). These enzymes shuttle amino groups between amino acids and their corresponding keto acids: AspAT acts on the dicarboxylic amino acids aspartate and glutamate; TyrAT acts on these two as well as the aromatic amino acids tyrosine, phenylalanine and tryptophan. The TyrAT protein has so far resisted all attempts to determine its structure, so Jansson and colleagues have taken an indirect approach to analyse the dual specificity of TyrAT. They determined the structures of various enzyme-inhibitor complexes of an AspAT hexamutant with a substrate specificity more typical of TyrAT. The two carboxylate groups of a dicarboxylic amino-acid substrate analogue form strong hydrogen bonds/ion pairs with the

side chains of Asp 386 and Arg 292\* (the asterisk indicating that the Arg 292 is from the second subunit in the homodimeric enzyme). The single carboxylate group of the aromatic substrate analogue makes a similar contact with Asp 386 and the phenyl ring is found in the same binding pocket as the second carboxylic acid group, except that the phenyl ring is wedged snugly between an indole ring and an aliphatic side chain. As a result, the Arg 292\* side chain is forced out of the binding pocket and into the surrounding solvent. It is thought that, in the wild-type enzyme, excess substrate-binding energy is used to drive the conversion of the dimeric

nature  
Structural  
biology



Turned turtle — arrangement of tetramers in phenylalanyl-tRNA synthetase.

enzyme from the open (ligand-binding) conformation to the closed (catalytic) conformation. The structure of the mutant enzyme without ligand is already in the closed conformation, suggesting that the energy of substrate binding could be used to expel the Arg 292\* side chain from the binding pocket and thereby allow the aromatic ring to bind in TyrAT. □

## DiGeorge syndrome breakpoint

DiGeorge syndrome (DGS) is one of the more severe forms of a group of genetic disorders that are all associated with deletions in the long arm of chromosome 22 and which between them make up a large source of birth defects. They have been given the acronym CATCH 22, reflecting their common characteristics: cardiac defect, abnormal facies, thymic hypoplasia, cleft palate, hypocalcaemia and chromosome 22 deletions. Patients with these disorders are

hemizygous for the genes in the deleted region, so the genetic defects may be caused by one or more genes being rendered haploinsufficient. Although deletion analysis can be a powerful tool for pinpointing a candidate disease gene, in DGS the region that is deleted in all patients is about 1.5 megabases long, which is too large to make correct gene selection likely. The discovery of a rare DGS patient, who instead of a deletion had a balanced

nature  
genetics

### Also in *Nature Structural Biology*

- Superantigen /MHC II interactions.
- $\beta$ -glycanase structures.
- DNA-repair enzyme active site.
- GroEL-substrate interactions.
- DNA-binding anticancer drug.
- Intrinsic amino acid  $\phi$ - $\psi$  propensities.

translocation with a breakpoint within the DGS critical region, provided the breakthrough scientists needed. A balanced translocation means that, unlike the more common, deletion-carrying DGS patients, this DGS individual is not hemizygous for a series of genes. Therefore, the gene or genes disrupted by the translocation breakpoint are the most likely candidates.

On page 269 of the July issue of *Nature Genetics*, M.L. Budarf and colleagues describe the cloning of the breakpoint region in this balanced translocation between chromosomes 2 and 22. In an accompanying News & Views, Thomas W. Glover describes the history of the search for the DiGeorge syndrome gene by these and other researchers.

After cloning out the breakpoint region, the researchers performed a sequence analysis of the cloned translocat-

ion breakpoint. They found that the gene *DGCR2/LAN* (previously considered to be a strong candidate for DGS) is unlikely to play a major role because its 3' end lies some 10 kilobases away from the disrupted region. Attention was turned instead to two open reading frames (ORFs) that span the breakpoint. One of these ORFs codes for a protein that has similarity to the murine androgen receptor and contains a leucine-zipper motif, suggesting that it could be a DNA-binding protein and might play a part in developmental regulation — but so far this is only speculation.

Budarf and colleagues strengthened the argument for a role in DGS for these two ORFs by showing that they are deleted in all of the 22 patients they examined. Further evidence will come from screening all DGS individuals with deletions for loss of these loci, and the few patients without deletions for mutations in these ORF.

DGS remains a complex disorder, not least because the size of the deletion is not indicative of the severity of the disease. This adds weight to the idea that one or more specific genes, when altered, are the principal perpetrators of this disorder. There is also strong evidence that the genetic background of an individual may play a part in determining the severity and form of the disorder. This point is raised by Budarf *et al.* when they compare the disease severity of the DGS individual carrying the balanced translocation with that of her mother, who also carries the balanced translocation. The mother of the proband, rather than having DGS, suffers from one of the related but milder CATCH 22 disorders, velocardiofacial syndrome. □

MAX to its normal partner MYC, or perhaps also by an active repression of transcription. In this paper, an adenoviral vector overexpressing MAD (AdMAD) was used to infect a human astrocytoma tumour cell-line (U-373MG) that expresses an unusually stable version of MYC. Cell growth, as measured by [<sup>3</sup>H]thymidine incorporation and cell-doubling time, was convincingly inhibited, and the transfected cells were also interrupted in the cell cycle and arrested in the G1 stage. To determine whether MAD could also affect the malignant potential of human tumour cells, U-373MG cells infected with AdMAD were examined both *in vitro* and *in vivo*. *In vitro*, infected cells showed a very significant reduction in their ability to form foci, as compared with control cells; and when nude mice were injected subcutaneously with infected or mock-infected U-373MG cells, the transfected (MAD-expressing) cells developed into tumours at less than one-tenth of the rate of mock-infected control cells.

This is the first report of a MAD-mediated inhibition of malignant potential and suggests that the introduction of AdMAD-type viruses into human tumours may be a feasible approach for gene therapy. However, it should be remembered that the potential use of this type of therapy is still in its infancy. Some tumour cell lines, such as those derived from ovarian cancer and Burkitt's lymphoma, are resistant to adenovirus infection. At the opposite extreme, it has not yet been established whether overexpression of MAD will adversely affect normal cells.

Finally, the tumour-rejection studies reported in this paper were not completely effective. Eleven mice were injected with AdMAD-infected U-373MG cells and followed for 25 days. In spite of the presence of the MAD gene product, one of these mice did develop a tumour. Nevertheless, malignant brain tumours, particularly the high-grade astrocytic tumours from which the U-373MG cell line was derived, are notoriously resistant to chemoradiotherapy, making these preliminary steps all the more exciting. □

#### Also in *Nature Genetics*

■ Mutations in the gene encoding cartilage oligomeric matrix protein are involved in pseudoachondroplasia, an inherited form of dwarfism, and in multiple epiphyseal dysplasia, a related disorder.

■ A gene that causes nocturnal enuresis, or nightly bedwetting, is localized to the long arm of chromosome 13.

■ Length of short repetitive DNA-sequence arrays in men versus apes suggests evolution of array size differs from one species to the next.

■ Completion of the yeast chromosome VI sequence puts the yeast genome project at 70% complete.

## MAD genes attack cancer cells

THE *myc* family of proto-oncogenes is thought to contribute to the regulation of cellular proliferation, and aberrant expression of these genes can result in overproliferation phenotypes such as neoplasms. MAD, a natural antagonist of the *myc* protein MYC, has been recently shown to hold MYC expression in check. Jun Chen *et al.* reasoned that, by overexpressing MAD, it might be possible to inhibit the proliferation of MYC-induced neoplastic cells. On page 638 of the July issue of *Nature Medicine*, they present *in vitro* and *in vivo* evidence to support this idea. Using an adenovirus to overexpress MAD in human brain tumour cells, they demonstrate an inhibition of growth and other malignant properties, laying the groundwork for future gene therapy approaches in the fight against tumours.

The normal role of MAD in the cell is

not completely understood, but a number of observations suggest that it may function as a growth/tumour suppressor; overexpression may inhibit proliferation and/or tumorigenesis, and inactivation may contribute to oncogenesis. MYC is known to bind the protein MAX, forming a heterodimer that interacts at a promoter-proximal 'E-box' to regu-

late transcription of genes involved in growth control. MAD was a promising antagonist because it was known

that it could also form heterodimers with MAX (as can a closely related antagonist, MX11) and that these MAD-MAX heterodimers compete with the MYC-MAX heterodimers for common target sites at promoters. In this manner, they can inhibit MYC-induced transactivation. MAD may also inhibit transactivation by binding and thus restricting the supply of

nature  
medicine

#### Also in *Nature Medicine*

■ SPECT scans of the brain reveal that the density and distribution of dopamine receptors is altered in alcoholics compared with healthy controls.

■ Heterozygosity for the most common cystic fibrosis mutation may confer a protective advantage against bronchial asthma.

■ PET scans define a migraine centre in the human brain stem.

■ The moral and legal ramifications of voluntary euthanasia.

# Amplification technology

Product review comes in loud and clear with a PCR detection system, a solid phase sequencing kit, a microdispensing system, a template isolation kit and a DNA surface decontaminant.

BOEHRINGER Mannheim has launched Split Second, a product designed to reduce exposure time and handling steps during **PCR preparation** (*Reader Service No. 100*). The manufacturer states that the product reduces the number of assay steps and takes just 30 min to perform, requiring only 5–10 min of 'hands on' time. The user performs all reaction steps in a single test tube; this is intended to reduce exposure to potentially hazardous material or infectious agents. Additionally, two proprietary solutions are provided, eliminating the use of chaotropic agents and the need for potentially hazardous extractions.

Life Technologies now offers eLongase reagents for the efficient **amplification of a variety of templates for long PCR** (*Reader Service No. 101*). These reagents contain an optimized mixture of recombinant, thermostable polymerases, including a proofreading (3'–5' exonuclease) activity, resulting in efficient amplification of long templates and high yield. The manufacturer states that the product allows amplifications up to 30 kb for lambda DNA, up to 20 kb for single-copy genomic DNA and amplification of long cDNA templates in RT-PCR applications.

Oncor's OptiProbe **tissue fixative**, optimized for use in tissue *in situ* hybridization and PCR, is a non-crosslinking fixative that is gentle on tissues and cells and requires no special handling or disposal (*Reader Service No. 102*). Manufactured to be RNase free, it is



Oncor's tissue fixative.

compatible with antibodies, DNA probes and mRNA. According to Oncor, tissue fixed with this product requires no predigestion or other recovery procedures before *in situ* hybridization. It is said to yield high-quality results in PCR.

Perkin-Elmer has developed the TaqMan LS-50B **PCR detection system** to make PCR a routine assay for quantitative sequence detection (*Reader Service No. 103*). This system employs a



TaqMan LS-50B from Perkin-Elmer.

5' nuclease assay incorporated into the reagent kit. It includes PCR primers and a fluorogenic probe that emits fluorescence only if the target sequence is amplified. The system consists of a reagent kit and the LS-50B luminescence spectrometer, and the manufacturer states that it can detect up to 96 samples in just 7 min. It has also been optimized for use with the GeneAmp PCR system 9600.

Kristal Clean KC is a new mini-prep kit for the **removal of primers and excess oligonucleotides after PCR** procedures and before cloning (*Reader Service No. 104*). It is manufactured by Molecular Technologies for the removal of small oligomers of DNA from modification reactions and the removal of unincorporated radioactive nucleotides after oligolabelling reactions. The kit provides a quick and simple protocol starting with the whole reaction mixture and utilizes Kristal size-exclusion resin and a spin column — this resin can also be used for the removal of linkers.

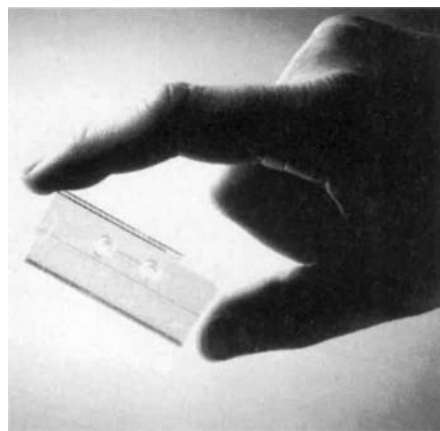
The new AmpliScript kit from Kalyx Bioscience has been designed to provide the researcher with enhanced sensitivity of **detecting nucleic acid amplification reaction products** in a standard microplate format. (*Reader Service No. 105*). This system is said to allow the user to enhance PCR sensitivity by converting each DNA amplicon molecule to numerous copies of RNA, which are subsequently detected by a simple hybridization and immunoenzymatic assay procedure. This format provides for colorimetric determination of the product.

## ■ Sequencing

Amersham Life Science has developed a new **cycle-sequencing system** based on ΔTaq version 2.0 DNA polymerase (*Reader Service No. 106*). Unlike Taq polymerase, ΔTaq has no associated 5'–3' exonuclease activity; this reduces background bands to a

minimum, dramatically improving base calling accuracy and readability. The manufacturer states that sequencing can be performed where only low levels of template are available. A two-stage labelling and termination protocol is provided, which is said to ensure a higher signal intensity and greater sequence quality, particularly close to the primer. In the labelling step, the template, primer and enzyme are mixed with two unlabelled dNTPs and one α-labelled dNTP. By omitting a specific nucleotide in this step, primer extensions can be carefully controlled so that labelled primers of known length are produced. As a result, sequencing reactions benefit from uniform labelling.

Pharmacia Biotech has designed the AutoLoad **solid-phase sequencing kit** to provide a simple and more convenient way of preparing samples for DNA sequencing (*Reader Service No. 107*). It is intended to improve the handling, processing and sequencing of biotinylated PCR products. The kit allows the direct elution of PCR products into the wells of sequencing gels from solid-phase, streptavidin-coated



Pharmacia's solid-phase sequencing kit.

combs. Samples are processed in parallel at every step of the process; this is intended to make ten sequencing reactions as easy to handle as one. Each comb carries two full reactions of ACGT and will efficiently capture PCR products up to 800 bp in length. The combs are optimized for use with 0.5 mm sequencing gels.

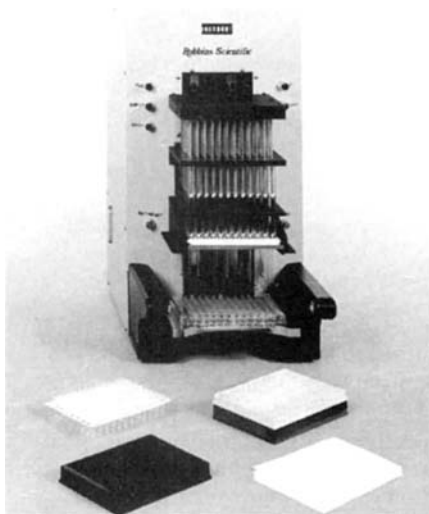
## ■ Mechanization

Robbins Scientific has introduced the Hydra-96 **microdispenser**, the first instrument to provide microlitre dispensing through 96 channels without the use of pipette tips (*Reader Service No. 108*). Constructed from 96 precision-glass



## PRODUCT REVIEW

syringes that fill and dispense in unison, the device delivers volumes as low as 0.5 µl per channel. All parameters are programmable and can be stored in 1 of 16 separate files. It can also be integrated into an automated laboratory system by interfacing with an external computer via an RS 232 port. The system can be used in



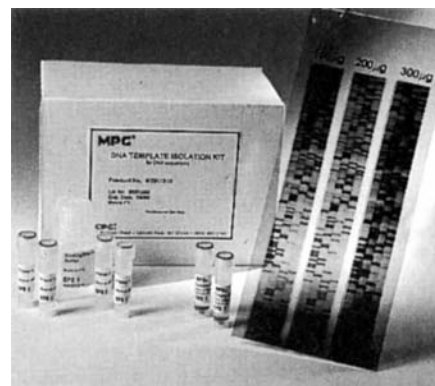
Robbins Scientific's microdispenser.

genome mapping and sequencing laboratories for preparing PCR and cycle-sequencing reactions.

Savant's Gene Roller **hybridization oven** features rotisserie movement and forced air circulation for uniform temperature within each bottle, and from bottle to bottle (*Reader Service No. 109*). The digital controller is calibrated to monitor internal solution temperature, for optimizing hybridization protocols. It has a temperature range from 30 °C to 85 °C, includes a safety shut-off and audible over-temperature alarm. The product's durable safety bottles, in conjunction with the polycarbonate door, shield 99 per cent of beta emissions. The stainless steel interior, removable drip tray (200-ml capacity) and immersible high-capacity rotisserie (10 bottles) are said to be simple to clean. A slipping rotisserie clutch is designed to permit safe, easy loading and removal of bottles during rotation. If desired, the rotisserie can be offset for maximum solution agitation.

### ■ Purification

New from CPG is the **MPG DNA template isolation kit**, which is designed to provide a reliable means of isolating DNA



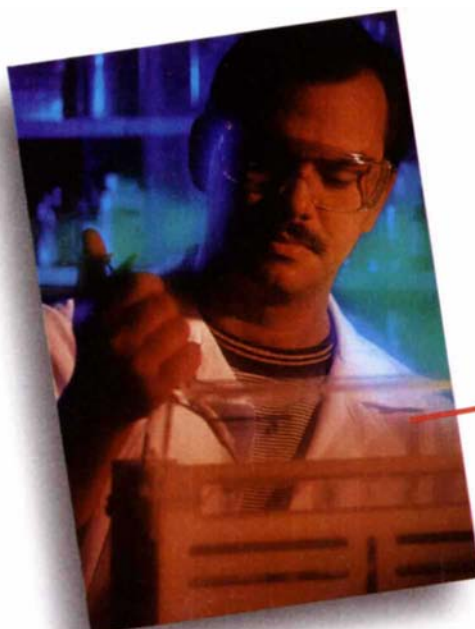
The MPG DNA template isolation kit.

templates for direct sequencing of PCR fragments (*Reader Service No. 110*). This is said to offer a higher probability of success in generating PCR sequencing results the first time without having to repeat reactions. These are porous, paramagnetic glass particles with a streptavidin coating, which are intended to have more applications than polystyrene magnetic particles allowing for less precise estimates of the quantity of target DNA. This product is said to use up to two-thirds fewer particles per isolation, thus lowering the cost per reaction. Results beyond 400-base-pair

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### Ron Lirette, Ph.D.

- Presently directing research activities and product development for Sigma BioSciences.
- Published in *Journal of Cellular Biochemistry*, *Journal of Virology*, *Journal of General Virology*, and *EMBO*.
- Presented abstracts at American Society for Cell Biology Conferences, Symposium of the Protein Society, Annual Meeting of The American Society for Virology, and International Symposium on Column Liquid Chromatography.
- Prior to Sigma, worked five years in a molecular biology group in the pharmaceutical industry.
- Also worked in academia at the Wistar Institute of Anatomy and Biology and at the University of Medicine and Dentistry of New Jersey.
- Received his Ph.D. from Louisiana State University.



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resolution can be obtained using either automated or manual sequencing methods. Biotinylated and non-biotinylated primers included with the kit enable PCR amplification and sequencing of both strands of the cloned DNA insert.

New from Life Sciences International is IEC's new economy **benchtop centrifuge**, which features sealed tube shields for aerosol containment, offering complete protection to users centrifuging potentially hazardous urine and blood samples (*Reader Service No. 111*). An added benefit of the new Medispin is the transparent window in its shield, which allows broken tubes to be detected without opening the seal, allowing decontamination if necessary. It accepts a

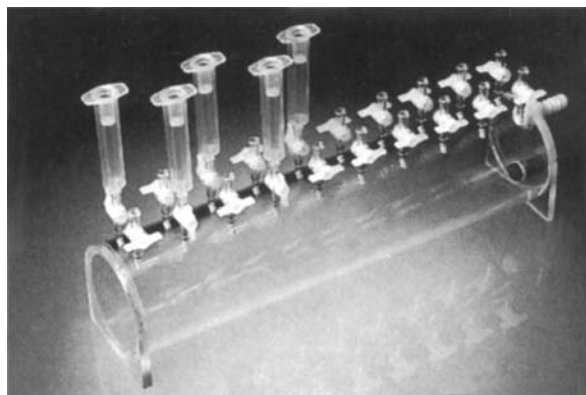


IEC's MediSpin bench-top centrifuge.

variety of different tube sizes — accommodating up to six 15-ml or twelve 10-ml tubes. Achieving a top speed of 3,100 r.p.m. and 1,228g, the centrifuge has an integral timer for programming runs of 0–300 min.

Promega has introduced the new Wizard 373 **DNA purification kit** for the rapid isolation of plasmid DNA for cycle sequencing with the Applied Biosystems 373A DNA sequencer (*Reader Service No. 112*). This kit can be used to isolate rapidly and efficiently around 2 µg of low copy number plasmids (four to six copies per cell), as well as 5–15 µg of standard high copy number plasmids. Using Microcon 100 microconcentrators, isolated DNA can be obtained at a consistent purity and concentration for automated sequencing. The kit can be used to process up to 20 samples of 1.5- to 10-ml culture volumes in less than 40 min.

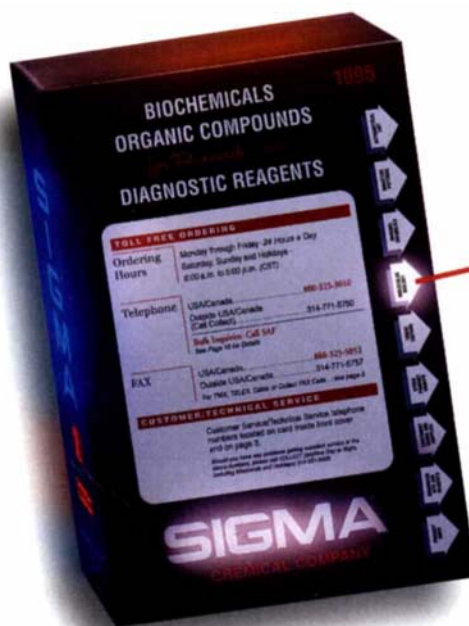
The GeniePrep DNA purification kit, from Ambion, has applications that



GenieVac from Ambion.

include plasmid mini-prep purification, purifying PCR products to remove primers and nucleotides, purifying restriction digested plasmid and PCR products and removing unincorporated nucleotides from labelled DNA (*Reader Service No. 113*). The GenieVac-20 allows from one to twenty 5-ml syringes to be attached to the manifold. The GeniePrep fibre pad cartridges fit into the syringes allowing up to 20 preps or samples to be processed simultaneously. The vacuum manifold is highly recommended over the centrifuge method for GeniePrep applications because it is

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## PRODUCT REVIEW

much faster and does not require emptying of microcentrifuge tubes or transfer of the GeniePrep fibre pad cartridges to empty microfuge tubes.

Sartorius Separation Technology's new disposable Centrisart-C4 microconcentrators are designed for **ultrafiltration or microfiltration of small volumes** (<0.4 ml) in a standard microcentrifuge (Reader Service No. 114). These microconcentrators are designed to ensure fast filtration rates, resulting in substantial



**Centrisart from Sartorius.**

time savings. There are two main parts: an insert tube for the sample with an attached cap and filter sealed in the base, and an outer centrifuge tube in which the filtrate is collected. The separate colour-coded cap of the centrifuge tube identifies the type of filter. They are designed to be a convenient alternative to precipitation, lyophilization and dialysis in DNA and protein work.

The latest release from Dynal is Dynabeads DNA Direct for biomagnetic separation of PCR-ready genomic DNA directly from blood, bone marrow and cultured cells (Reader Service No. 115). This product is designed to eliminate centrifugation steps and avoid the use of hazardous chemicals. A 10-min, single-tube procedure can produce PCR-ready genomic DNA from 10 µl of blood. The manufacturer states that this product is equally suited for laboratories handling small, precious samples or for those with high-throughput requirements. It is available in two sizes: an introductory 100 sample test kit and a larger 250 sample test kit.

### ■ Miscellaneous

Polyfiltronics has developed an international footprint **thin-wall PCR plate** with a thick wall frame similar to standard microplates for use with PCR thermal cyclers (Reader Service No. 116). Designed to be robotic-friendly, this 96-well PCR plate has a rigid frame that enables pick-up without the flexing associated with vacuum-formed PCR plates. Each well extends beyond the surface allowing for an around-the-well seal with a variety of thermal cyclers. Alternatively 8- and 12-well cap strips are also available.

Molecular Bio-Products has introduced a new **surface decontaminant** that will help ensure PCR workstations, countertops and apparatus are free from DNA contamination (Reader Service No. 117). DNA Away surface decontaminant is designed to eliminate all DNA from glassware and plasticware without having a residual effect on subsequent DNA samples. The manufacturer states that it is more effective for degrading DNA than autoclaving.



**DNA Away.**

The Socorex Stepper 411 has been designed to be a comfortable, **adjustable repeat pipette**, which covers a range from 10 to 5000 µl (Reader Service No. 118). The manufacturer states that only three Ecostep tip sizes are necessary for a total of 53 different volumes. Three colour-coded volume setting knobs, which correspond to tip colour for identification, show actual volume set and number of aliquots delivered. The drive unit stops automatically if the last aliquot cannot be delivered in full. Tips are supplied in packs of 100. □

*These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal. The PCR process is covered by patents owned by Hoffman-LaRoche.*

### Correction

In the product review article "Fluorescence polarization — a new tool for cell and molecular biology" (*Nature* 375, 254-256; 1995), LeTilly and Royer were erroneously cited. The reference should have been Lee, J. & Heyduk, T. *Proc. natn. Acad. Sci. U.S.A.* 87, 1744-1748 (1990).

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